Molecular and Enzymatic Characterizations of Novel Bifunctional 3β-Hydroxysteroid Dehydrogenases/C-4 Decarboxylases from Arabidopsis thaliana

Received for publication, May 9, 2006, and revised form, June 30, 2006. Published, JBC Papers in Press, July 11, 2006. DOI 10.1074/jbc.M604431200

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We have isolated two cDNAs from Arabidopsis thaliana encoding bifunctional 3β-hydroxysteroid dehydrogenase/C-4 decarboxylases (3βHSD/D) involved in sterol synthesis, termed At3βHSD/D1 and At3βHSD/D2. Transformation of the yeast ergosterol auxotroph erg26 mutant, which lacks 3βHSD/D activity, with the At3βHSD/D1 isoform or with At3βHSD/D2 isoform containing a C-terminal At3βHSD/D1 endoplasmic reticulum-retrieval sequence restored growth and ergosterol synthesis in erg26. An in vitro enzymatic assay revealed high 3βHSD/D activity for both isoenzymes in the corresponding microsomal extracts. The two At3βHSD/D isoenzymes showed similar substrate specificities that required free 3β-hydroxyl and C-4-carboxyl groups but were quite tolerant in terms of variations of the sterol nucleus and side chains. Data obtained with 4α-carboxycholest-7-en-3β-ol and its 3α-deuterated analog revealed that 3α-hydrogen-carbon bond cleavage is not the rate-limiting step of the reaction. In planta reduction on the expression of the 3βHSD/D gene as a consequence of VIGS-mediated gene silencing in Nicotiana benthamiana led to a substantial accumulation of 3β-hydroxy-4β,14-dimethyl-5α-ergosta-9β,19-cyclo-24(241)-en-4α-carboxylic acid, consistent with a decrease in 3βHSD/D activity. These two novel oxidative decarboxylases constitute the first molecularly and functionally characterized HSDs from a short chain dehydrogenase/reductase family in plants.

Sterols are essential components of all eukaryotic cell membranes, and the biosynthetic pathways differ significantly. The sterol molecule becomes functional only after removal of the two methyl groups at C-4. Both methyl groups at C-4 are removed early and successively in animals and yeast, whereas in higher plants one methyl group is initially removed from a 4,4-dimethyl-9β,19-cyclopropylsterol precursor, and the second is eliminated several steps later (1–3). In plants, we have characterized the activities of a sterol C-4 methyl oxidase (SMO), a 4α-carboxysterol-3β-hydroxysteroid dehydrogenase/C-4 decarboxylase (3βHSD/D), and an NADPH-dependent 3-oxosteroid reductase from partially purified preparations (4–6) in order to define the steps involved in C-4 demethylation in plants. The first step is initiated by the SMO, whereby this enzyme converts the C-4α methyl group to produce a 4α-carboxysterol derivative that is subsequently oxidatively decarboxylated by the 3βHSD/D to produce a C-4-monodemethylated 3-oxosteroid, which is then stereospecifically reduced by the 3-ketoreductase. In contrast to animals and yeast where the SMO is encoded by a single gene (7), we biochemically characterized two distinct microsomal SMOs in Zea mays (4), and we identified two distinct families of SMO genes in Arabidopsis thaliana (8). Until now, the gene(s) coding the single bifunctional protein 3βHSD/D (Fig. 1) have not been characterized.

Furthermore, little is known about plant hydroxysteroid dehydrogenases that are either members of the short chain dehydrogenases/reductases (SDR) (9–11) or the aldo-keto reductase family (12), which include soluble and membrane-bound HSDs. Identification and characterization of gene(s)-encoding plant HSD of the SDR family have not been reported thus far.

To further our knowledge about hydroxysteroid dehydrogenases and the sterol C-4 demethylation multienzymatic complex in plants, in particular, we have identified and characterized at the molecular and enzymatic levels two novel 3βHSD/D isoforms from A. thaliana. In this study, we present identification and cloning of these 3βHSD/Ds cDNAs, in vivo heterologous and functional expression in yeast, in vitro enzymological characterization of the recombinant isoenzymes, and in planta down-regulation by virus-induced gene silencing.

EXPERIMENTAL PROCEDURES

Materials

All materials were purchased from Sigma or as otherwise specified in the text.

Strains and Plasmids—The erg26 SDG200 strain of Saccharomyces cerevisiae, deficient in 3β-hydroxydehydrogenase/C-4 decarboxylase activity (Mata ade5, his3, leu2-3, ura3-52, 3βHSD/D, 3β-hydroxysteroid dehydrogenase/C-4 decarboxylases; ER, endoplasmic reticulum; SDR, short chain dehydrogenases/reductases; HSD, hydroxysteroid dehydrogenase; GC-MS, gas chromatography-mass spectrometry; RT, reverse transcription.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. A–D.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) AY957470, DG302749, AM226957, and AM226957.

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2 Supported by National Institutes of Health Grant GM62104.
erg26Δ::TRP1 trp1::hisG Δhem1) used in the present study, has been described previously (13). Sterol auxotrophs were grown aerobiologically at 30 °C on solid enriched medium (YPG: 1% yeast extract, 2% peptone, 2% glucose) supplemented with 2% ergosterol or cholesterol dissolved in ethanol/Tween 80 (1:1, v/v) or on minimal medium (YNB: 0.67% yeast nitrogen base, 2% glucose) containing suitable supplements (50 mg/liter each), casamino acids (1 g/liter) and 2% of ergosterol or cholesterol. In the case of liquid medium, the concentration of sterol used was 0.5%. Sterol prototrophic strains were grown aerobiologically at 30 °C on solid or liquid YNB medium containing suitable supplements (50 mg/liter each) or enriched medium (YPG) in the presence of δ-aminolevulinic acid (50 mg/liter).

The pVT102U (14) S. cerevisiae shuttle vector optimized for expressing recombinant proteins in yeast was used for cloning, sequencing, and transformation of the erg26 strain. This plasmid contains an Escherichia coli origin of replication, a yeast 2-µm origin of replication, an E. coli ampicillin resistance gene, and the yeast URA3 gene. It contains an expression cassette, including the alcohol dehydrogenase promoter and terminator. TTO vector is an RNA viral vector and has been described previously (15). It consists of sequences from tobacco mosaic virus strain U1 (TMV-U1) and tomato mosaic virus (fruit necrosis strain F; ToMV-F).

Plants—Nicotiana benthamiana was grown in a greenhouse at 24 °C with a 16-h light/8-h dark cycle. A. thaliana plantlets were from the Wassilewskija ecotype.

cDNAs Cloning—We searched the Arabidopsis genome for genes encoding putative orthologs of the yeast ERG26 gene and also belonging to the hydroxysteroid dehydrogenase (HSD) family. Two genes were selected as follows: At1g47290 and At2g26260, and their corresponding cDNAs (At3βHSD/D1 and At3βHSD/D2, respectively) were cloned by PCR using reverse-transcribed mRNAs from the Wassilewskija ecotype. The amplified cDNA fragments were cloned into the XbaI and Xhol sites of the pVT102U shuttle vector and placed under the control of the constitutive alcohol dehydrogenase promoter. Because the lack of a C-terminal ER retrieval signal KXXX in At3βHSD/D2 might result in inefficient localization in the ER yeast C-4 demethylation complex, we replaced the seven terminal amino acids of At3βHSD/D2 with the KKID sequence to produce Δ-At3βHSD/D2, which was also cloned into the pVT102U vector.

Total RNAs from A. thaliana plantlets were extracted using the TRizol reagent (Invitrogen) according to the manufacturer’s protocol and treated with DNase to remove any residual DNA. SuperScript II kit (Invitrogen) was used to perform RT-PCRs with the following reverse primers: 5′-TTAGCTGATCCTTCTGCTCCGAACACTTTC-3′ (P2) corresponding to At1g47290 (NM_179448) and 5′-CTAATTGAAATATATGGTGCA-TACCCTTCCGCCC-3′ (P4) corresponding to At2g26260 (NM_128183.1), to specifically amplify the corresponding cDNAs. PCR amplification using an aliquot of RT-PCR, with the primer pair P1 = 5′-ATGGAAGTTACAGAGACTGAGCG-

FIGURE 1. Reaction catalyzed by 3β-hydroxysteroid dehydrogenase/C-4 decarboxylases and putative mechanism.
**Plant 3β-Hydroxysteroid Dehydrogenase/Decarboxylase**

**Nicotiana.** A 500-bp fragment of the *N. benthamiana* 3β-hydroxysteroid-dehydrogenase-C-4 decarboxylase gene-amino acid residues from the C terminus was (Nb3HSD/D) (AM236597) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase gene (DXR) (AM236596), which was used as a control, were PCR-amplified from RNAs extracted from leaves of *N. benthamiana* using the following forward primers, respectively: 5′-GCCCTAGGCTGATTTGGGTCCATCATTTAATCGG-3′, 5′-GCCCTAGGCTGATTTGGGTCCATCATTTAATCGG-3′, and the following reverse primers: 5′-TCTTATTGCTGATTTGGGTCCATCATTTAATCGG-3′, 5′-TCTTATTGCTGATTTGGGTCCATCATTTAATCGG-3′, and 5′-TCTTATTGCTGATTTGGGTCCATCATTTAATCGG-3′. These products were cloned into the Xhol-AvrII restricted *TTO* viral vector (16) to generate *TTO-Nb3HSD/D* and *TTO-NbDXR*, respectively.

**Virus-induced Gene Silencing in N. benthamiana—** *TTO-Nb3HSD/D* and *TTO-NbDXR* constructs were used to inoculate young *N. benthamiana* plants as described previously (16). To measure silencing of *Nb3HSD/D*, semi-quantitative reverse transcription was performed also as described previously (8, 17). Total RNAs isolated from uninoculated *TTO-NbDXR* and *TTO-Nb3HSD/D* leaves were extracted and used for PCR. The forward 5′-AGAGAGGCTTICGCTGCTCG-3′ and reverse 5′-GCATGTGCTACATTCTCCACG-3′ primers that anneal outside the region used for PCR were used for PCR. The forward 5′-AGAGAGGCTTICGCTGCTCG-3′ and reverse 5′-GCATGTGCTACATTCTCCACG-3′ primers that anneal outside the region used for PCR were used for *Nb3HSD/D* silencing were employed. Amplification of the *N. benthamiana* α-tubulin gene was performed as a control using the forward 5′-ATGCTTTATCATATGCCCCGTG-3′ and reverse 5′-CAGCACAACAGTCTCTCGTAATC-3′ primers.

**Identity Scores and Phylogenetic Analysis—** Protein sequences of *Arabidopsis* 3βHSD/D and their homologs were identified by BLAST searches and aligned using the ClustalW algorithm. A neighbor-joining tree was constructed based on the sequence alignment and tested further with 1000 bootstrap resampling by using the MacVector package.

**Transformations—** *S. cerevisiae* transformations were performed using the lithium acetate procedure as described previously (18). The transformed erg26 yeast strain was plated on minimal YNB medium containing suitable supplements (adeneine, 50 μg ml⁻¹) without uracil and 2% of ergosterol, as well as on the same minimal YNB medium without uracil but also containing 6-aminolevulinic acid (50 mg/liter). Cells were grown aerobically at 30 °C.

**Sterol Analysis—** Lyophilized yeast cells (10–30 mg) were sonicated in the presence of KOH/methanol (6%, w/v) (2 ml) for 10 min and heated in the same medium at 70 °C under reflux conditions for 2 h. The mixture was diluted with 1 volume of water, and after acidification to pH 3, total sterols were extracted three times with 3 volumes of ethyl acetate. The extract was dried on Na₂SO₄, evaporated to dryness, treated with 500 μl of a 0.4 M solution of diazomethane in diethyl ether for 1 h at 0 °C and 1 h at room temperature, and then evaporated to dryness. Sterols were analyzed by gas chromatography. GC analysis was carried out with a Varian GC model 8300 (Les Ulis, France) equipped with a flame ionization detector at 300 °C, column injector at 250 °C, and a fused capillary column (WCO, 30 m x 0.25 mm inner diameter) coated with DB1 (H₂ flow rate of 2 ml/min). The temperature program used included a 30 °C/min increase from 60 to 240 °C and followed by a 2 °C/min increase from 240 to 280 °C. Relative retention times (tᵣ) are given with respect to cholesterol (tᵣ = 1). Identification of individual sterols was performed using a GC-MS spectrometer (Agilent 5973N) equipped with an “on column” injector and a capillary column (30 m x 0.25 mm inner diameter) coated with DB5. Sterols were unequivocally identified by retention times and an electron impact spectrum identical to that of authentic standards (19).

Lyophilized plantlets of *N. benthamiana* (0.9–2.0 g) were homogenized with an Ultra-Turrax homogenizer in the presence of methanol/methylene chloride (2:1, v/v) and heated in the same medium at 70 °C under reflux conditions for 3 h. The mixture was filtered, and the extract was evaporated to dryness. Acidic derivatives (Rₑ = 0.0–0.12), desmethylsters (Rₑ = 0.27), 4α-methylstereol (Rₑ = 0.38), 4,4-dimethylstereol (Rₑ = 0.44), and sterol esters (Rₑ = 0.70) were purified twice by TLC on Silica Gel 60F254 plates (Merck), using methylene chloride as developing solvent, and various fractions were eluted from the silica gel. Sterol esters were saponified under standard conditions. The polar fraction (Rₑ = 0.0–0.12) was treated with diazomethane at 0 °C and subsequently acetylated under standard conditions. The derivatized polar fraction was further purified by TLC on Silica Gel 60F254 plates (Merck), using methylene chloride as developing solvent. The fraction with an Rₑ = 0.07–0.20 and migrating with the standard of 4-carboxymethylsterol acetate was eluted from the silica gel. A known amount of cholesterol was added as internal standard for GC quantification in the case of the 4α-methylsterol, 4,4-dimethylsterol, and 4-carboxymethylsterol acetate fractions and of coprostane in the case of the desmethylsterol fraction. All fractions were then analyzed by GC and GC-MS under the same conditions as described above.

**Chemical Details—** Melting points are uncorrected. Proton magnetic resonance was monitored in a [1H]chloroform solution with a Brucker 400- or 500-MHz spectrometer. Chemical shifts (δ) (ppm) were determined relative to tetramethylsilane. Coupling constants (J) were in Hertz.

**Substrates—** For 4α-carboxy-cholest-7-en-3β-ol (1) (Fig. 2) was synthesized as described previously (5). The melting point was 231–233 °C; MS m/z (relative intensity) M₊ = 430(36), 412(10), 397(5), 386(100), 317(2), 299(19), 273(19), 271(19), 255(63), 229(22), 213(18). For 1H NMR: δ 0.533 (3H, s, H18), 0.844 (3H, s, H19), 0.862 (3H, d, J = 6.6, H26 or H27), 0.867 (3H, d, J = 6.6, H26 or H27), 0.918 (3H, d, J = 6.5, H21), 2.030 (3H, dt, J = 11, J = 4, H6β), 2.364 (1H, dd, J = 11, H6β), 3.809 (1H, m, H3α), 5.131 (1H, S, ω1/2 = 10, H7).

For synthesis of 3α-deutero-4α-carboxy-cholest-7-en-3β-ol (11), a solution of 4α-carboxymethoxycholest-7-en-3-one (120 mg) (synthesized as described previously (5) in absolute methanol (20 ml)) was stirred with sodium deuteroxoborohydride (NaBD₄) (Aldrich) (25 mg) for 2 h at 0 °C. After the usual work up, the solid residue was purified by TLC (SiO₂, eluted with ethene/ethyl acetate, 75:25 v/v) to yield pure 3α-deutero-4α-carboxymethoxycholest-7-en-3β-ol (Rₑ = 0.18) (55 mg), which was then separated from its 3α-hydroxy epimer (Rₑ = 0.44) and crystallized in methanol, m.p. 147–149 °C. The compound showed a single peak in GC (tᵣ = 1.368, DB5). MS m/z (relative...
Synthesis of 4α-carboxy-cholest-7-en-3β-ol (10) and 4α-carboxy-cholest-7-en-3α-ol (5). Reduction of 4α-carboxymethoxy-cholest-7-en-3-one by sodium borohydride (NaBH₄) and TLC purification under the same conditions as described above yielded 4α-carboxymethoxy-cholest-7-en-3β-ol (10) ($R_f = 0.18$) and 4α-carboxymethoxy-cholest-7-en-3α-ol (21) ($R_f = 0.44$). (10) was crystallized from methanol as follows: m.p. 152–154 °C. It showed a single peak in GC ($t_R = 1.368$, DB5). MS m/z (relative intensity) $M^+ = 444(41)$, 426(64), 411(46), 384(69), 367(12), 351(100), 313(66), 271(31), 253(22). For $^1$H NMR: δ: 0.529(3H, s, H18), 0.833(3H, s, H19), 0.863(3H, d, J = 6.6, H26 or H27), 0.868(3H, d, J = 6.6, H26 or H27), 0.917(3H, d, J = 6.5, H21), 2.341(1H, dd, J = 10.3, J = 10.3, H4b), 3.719(3H, s, COOCH₃), 3.802(1H, m, H3), 5.111(1H, d, J = 3.7, H7). Compound (21) showed a single peak in GC ($t_R = 1.333$, DB5). MS m/z (relative intensity) $M^+ = 444(100)$, 426(37), 411(31), 384(27), 367(14), 351(76), 313(56), 271(19), 253(22), 211(29). Saponification of (21) with KOH in ethanol/H₂O showed a single peak in GC ($t_R = 1.334$, DB5); MS: m/z (relative intensity) $M^+ = 444(100)$, 426(45), 411(40), 384(23), 367(10), 351(79), 313(55), 271(25), 253(22), 21(18).

For 4α-carboxy-3β-methyl-cholest-8,24-dien-3β-ol (2), this carboxysterol was extracted and purified from the erg26 yeast strain growing on YPG-enriched liquid medium supplemented with 0.5% of ergosterol and in the presence of δ-aminolevulinic acid (50 mg/liter) following the standard procedures described above. Crystallization in ethyl acetate yielded pure 4α-carboxycholest-7-en-3α-ol (5). 1H NMR of (5): δ: 0.534(3H, s, H18), 0.838(3H, s, H19), 0.865(3H, d, J = 6.6, H26 or H27), 0.871(3H, d, J = 6.6, H26 or H27), 0.922(3H, d, J = 6.5, H21), 2.507(1H, dd, J = 12.0, J = 2.2, H4b), 4.194(1H, s, $\omega^{1/2} = 8$ Hz, H3β), 5.150(1H, d, J = 3.3, H7). The 4α-carboxymethoxy derivative of (5) showed a single peak in GC ($t_R = 1.334$, DB5); MS: m/z (relative intensity) $M^+ = 444(100)$, 426(45), 411(40), 384(23), 367(10), 351(79), 313(55), 271(25), 253(22), 21(18).

For [24$^1-3$H]4α-carboxy-4β,14α-dimethyl-ergosta-9β,19-cyclo-24(24$^1$)-en-3β-ol (3) (1 μCi, 2.5 Ci/mol) and [24$^2-3$H]4α-
TABLE 1
GC-MS analysis of enzymatic products by the recombinant 3βHSD/Ds from Arabidopsis

| Substrate | Enzymatic product | GC(DB1) | MS (% relative abundance of molecular and prominent fragment ions) |
|-----------|-------------------|---------|---------------------------------------------------------------|
| 4α-Carboxy-cholest-7-en-3β-ol (1) and 4α-carboxycholest-7-en-3α-ol (5) | Cholest-7-en-3-one (6) | 1.074 | M+ = 384(100); 369(39); 271(68); 229(44); 213(17) |
| 4α-Carboxy-β-methyl-cholest-8,24-dien-3β-ol (2) | 4α-Methyl-cholest-8,24-dien-3-one (7) | 1.141 | M+ = 396(92); 381(100); 365(22); 283(22); 243(38) |
| 4α-Carboxy-7α,14α-dimethyl-9β,19-cycloergost-24(24)-en-3β-ol (3) | 4α,14α-Dimethyl-9β,19-cycloergost-24(24)-en-3-one (8) | 1.262 | M+ = 424(83); 409(61); 381(100); 340(39); 325(33); 300(33); 299(66) |
| 4α-Carboxy-stigmastera-7,24(24)-dien-3β-ol (4) | Stigmasta-7,24(24)-dien-3-one (9) | 1.306 | M+ = 410(3); 312(20); 297(10); 260(100); 229(8) |

carboxy-5α-stigmastera-7,24(24)-dien-3β-ol (4) (2 µCi, 2.5 Ci/mol), these compounds were prepared and purified as described in Ref. 4 by enzymatic 4α-methyl oxidation of [242,3H]4-methylenecycloartenol (13) and [242,3H]24-ethylideneolophenol (14), respectively, using a maize microsomal extract in the presence of NADPH. They showed a single radioactive band on SiO2–TLC using ethyl acetate as developing solvent.

[242,3H]4,4,14α-trimethyl-9β,19-cycloergost-24(24)-en-3β-ol (24,24-dimethyleneolophenol) (13) was enzymatically synthesized and purified as described in Ref. 4. They showed a single peak in GC and a single radioactive band on SiO2–TLC using methylene chloride as developing solvent. They were diluted with cold material to the desired specific radioactivity (2–5 Ci/mol). [13C4]4,4-Dimethyl-cholest-8,24-dien-3β-ol (12) was isolated from the mutant erg25-25c grown in the presence of [2,14C]acetate as described in Ref. 20 and showed more than 98% GC and TLC purity. Specific radioactivity was 1.5 Ci/mol.

Pregnenolone (16), trans-androstenedione (17), 3β-androstenediol (18), and dehydroepiandrosterone (19) were purchased from Sigma.

Preparation of Microsomes—Yeast microsomes were prepared as described previously (20). The corresponding 100,000 × g supernatants corresponding to the cytosolic extracts were concentrated 5–8-fold by dialysis over carboxymethylcellulose sodium salt (Fluka) for 16 h at 4 °C.

Standard Assay for Recombinant 4α-Carboxysterol-3-dehydrogenase-C-4 Decarboxylases—Microsomes (0.8 mg of protein) were incubated in the presence of exogenous synthetic 4α-carboxy-5α-cholest-7-en-3β-ol (1) (20–200 μM) emulsified with Tween 80 (final concentration 1.5 g/liter) and NAD+ at a 400 μM concentration (which is 50-fold the Km value). Incubations were continued aerobically at 30 °C with gentle stirring for 30–45 min. During this period the progression of the reaction was linear. The reaction was stopped by adding 0.5 ml of EtOH. After addition of a known amount of coprostanone (1–4 μg) as internal standard, sterols and sterones were extracted from the incubation mixture three times with a total volume of 15 ml of n-hexane, and after drying with Na2SO4, the extract was concentrated to dryness. Under these conditions, the residual carboxysterol substrate was not extracted. The extract was analyzed by TLC on silica gel eluted with CH2Cl2 (developed twice). The fraction migrating as authentic standards of coprostanone and cholest-7-en-3-one (6) and containing the enzymatically produced cholest-7-en-3-one (Rf = 0.50) was eluted and analyzed by GC (DB1) (supplemental Fig. A). The amount of cholest-7-en-3-one (6) produced (tR = 1.074) was calculated by comparison of the integrated peak areas with a known amount of added coprostanone (tR = 1.000), which also allowed the reaction rate of cholest-7-en-3-one to be determined. No endogenous component having the same tR as (6) was present in the inactivated control. The ketone metabolite produced by the reaction was unequivocally identified by its retention time on GC and by an electron impact mass spectrum identical to that of an authentic synthetic standard (Table 1 and supplemental Fig. A). Moreover, in control experiments, GC-MS analysis of the complete extract before TLC analysis indicated the absence of formation of cholest-7-en-3β-ol during the course of the incubation, confirming that in the absence of exogenously added NADPH to the microsomal extract, the C-4-demethylated-3-oxo-derivative was not reduced and further metabolized in accord with the cofactor requirements for this reduction step (6). Under these conditions, the estimated limit of detection of the 3βHSD/D activity was 0.1–0.2 nmol × h−1 × mg−1. Incubation of the 3α-deuterated substrate (11) was performed under the same standard conditions. For control experiments, the untransformed substrate (11) was extracted from the incubation mixture with ethyl acetate and methylated with diazomethane. GC-MS analysis of the 4-carboxomethoxy derivative revealed no loss of deuterium, thus excluding a possible washout of the deuterium label at C-3 during the incubation procedure.

Apparent maximum velocity V and V/K values were determined by fitting the data to the Michaelis-Menten equation using the nonlinear regression program DRN-EASY derived by Duggleby which corresponds to Ref. 21. Computer-assisted linear regression analysis gave correlation coefficients greater than 0.98 (n = 5–6). The primary deuterium kinetic isotope effects were defined as D/V = H(Hmax/Vmax)/D(Vmax) and D(V/K) = H(Vmax/Km)/D(Vmax/Km) according to the conventions of Northrop (22, 23).

Incubation of 4α-Carboxysterol Analogos and Identification of Enzyme-generated Products—The apparent Km and Vmax of analogs (1, 5, and 2) (Tables 3 and 4) were determined by incubating them for 30–45 min at 30 °C under standard assay conditions in a microsomal preparation containing either 3βHSD/D1 or Δ3βHSD/D2. The concentration of substrate was 20–150 μM, and the concentration of NAD+ was 0.4 mM (50-fold the measured Km value). As indicated previously, in the absence of NADPH addition to the microsomal extract, the expected C-4-demethylated 3-oxo-derivative metabolites were
not further metabolized. In the case of substrates (3 and 4) for which apparent $K_m$ and $V_{max}$ values could not be determined because of difficulties in obtaining sufficient quantities of these two substrates, the reaction rates of (3 and 4) were compared with those of (1, 2, and 5) in microsomal extracts containing either 3βHSD/D1 or Δ3βHSD/D2. Low substrate concentrations (40 μM) were used to determine enzyme specificity; the concentration of NAD$^+$ was 0.4 mM, and the reaction rates measured for (2, 3, 4, and 5) were normalized to the rate obtained with the standard substrate (1) (taken as 100%, Fig. 4, A and B). In the case of the carboxysterols (2, 3, 4, and 5) after addition of coprostanone as internal standard, extraction, and TLC analysis, the fraction migrating between a standard of 4-demethyl-sterone (Rp = 0.50) and 4,4-dimethyl-sterone (Rp = 0.70) was eluted and analyzed by GC and GC-MS (Table 1). The 3βHSD/D products were unequivocally identified by their retention time and an electron impact spectrum identical to that of authentic standards (Table 1). The data were compared with those obtained in the case of a control where the microsomes were inactivated. In addition, in the case of incubation of radiolabeled carboxysterols (2, 3, 4, and 5), the newly labeled sterone was directly visualized by radioscanning of the TLC plate, in accord with the migration of the corresponding nonlabeled standards. The areas of the GC peak of coprostanone and complete recovery of these compounds, which was confirmed from endogeneous components of the same $t_R$ (if present) determined in the corresponding control, allowed the rate of transformation of these analogs to be measured. Incubations of labeled sterols (12, 13, and 14) and corresponding control incubation with inactivated enzyme were extracted according to the same procedure. Radioscanning of the TLC analysis indicated the complete recovery of the added substrate and absence of metabolic alteration of these compounds by any of the two recombinant At3βHSD/Ds microsomal preparations. In the case of the carboxymethyalted analog (10), the hexane extracts of the incubation and control samples were directly analyzed by GC and gave identical profiles, indicating the absence of detectable At3βHSD/D activity with this analog.

Similarly, the extracts of incubations of 3β-hydroxysteroids (16, 17, 18, and 19) and ursoic acid (15) and corresponding controls were directly analyzed by GC and GC-MS. Comparison to the corresponding controls performed with inactivated microsomes revealed the absence of dehydrogenated product and complete recovery of these compounds, which was confirmed by ion monitoring that corresponded to the masses of the substrate and the expected C-3 dehydrogenated product.

**Miscellaneous**—Membrane protein was determined as described by Bradford (24).

**RESULTS**

**Cloning of 3βHSD/D in Arabidopsis**—We searched the Arabidopsis genome for genes encoding putative orthologs of the yeast ERG26 gene and belonging to the HSD family. Among the best candidates were At1g47290 and At2g26260, two genes that shared the highest sequence identity with the yeast 3βHSD/D protein Erg26p (30 and 29% respectively). Corresponding cDNAs (named At3βHSD/D1 for A. thaliana 3β-hydroxy-steroid dehydrogenase/decarboxylase isoform 1 and At3βHSD/D2, respectively) were cloned by PCR using reverse-transcribed mRNAs from the Wassilewskija ecotype. Sequencing of At3βHSD/D1 (AY957470) indicated an open reading frame of 1143 bp with two nucleotide changes from the At1g47290 gene transcript (ecotype Columbia, NM_179448) leading to two amino acid changes. Sequencing of At3βHSD/D2 (DQ302749) indicated an open reading frame of 1173 bp that was identical with the annotated At2g26260 gene transcript (NM_128183.1). While this study was in progress, an additional possible splicing site of the At2g26260 gene leading to a 3′-extended sequence (1695 bp) was annotated (NM_128183.2); however, we were not able to amplify the corresponding cDNA from our RNAs preparation.

**Sequences Analysis**—At3βHSD/D1 (381 amino acids) and At3βHSD/D2 (391 amino acids) proteins share 80% identity (supplemental Fig. B). A sequence comparison analysis revealed significant homology between the two At3βHSD/Ds to the 3β-hydroxysteroid dehydrogenase/isomerase protein family (Pfam 01073), a member of the SDR superfamily (FAD/NAD(P)-binding Rossmann fold superfamily clan) (9–11, 25, 26). However, there is little sequence identity (only 15–30%) between different SDR enzymes. The two sequences include N-terminal conserved glycine and aspartic residues, TGGXXGXXAXX$_{18}$D, which are required to form the coenzyme-binding site. In addition, the At3βHSD/D1 and At3βHSD/D2 proteins possess the YXX$_{12}$K motif (159YXX$_{12}$K for At3βHSD/D1) conserved in the active site of most of the members of the SDR family (10) (supplemental Fig. B). Finally, a conserved serine residue, found at position 130 or 131 for At3βHSD/D1, might constitute the third residue of the catalytic triad proposed for a variety of members of the SDR family (9, 10, 27). In the At3βHSD/D1 and At3βHSD/D2 proteins, there is a single putative membrane spanning domain near the C terminus (supplemental Fig. B). Finally, At3βHSD/D1 possesses a C-terminal ER retrieval signal KKXX, which is absent in At3βHSD/D2.

**Phylogenetic Analysis of the At3βHSD/D**—A molecular phylogenetic tree of the amino acid sequences of a variety of characterized HSD proteins from different organisms related to the putative At3βHSD/Ds was designed (Fig. 3). The protein sequences of At3βHSD/D1 and At3βHSD/D2 show 30% identity with the yeast ERG26 protein (13) and 37% identity with the NAD(P)H steroid dehydrogenase-like protein from animals (28). These clones show 22% identity with the human 3β-hydroxysteroid dehydrogenase/isomerase (29–30) and 26% identity with the cholesterol dehydrogenase from Nocardia (31), all enzymes catalyzing dehydrogenation of 3β-hydroxysteroids. The similarity of the two At3βHSD/Ds to Erg26p and Nsdhl is higher than with any other known 3βHSD. Together with the present 3βHSD/Ds from plant, these orthologs from yeasts and animals form a differentiated cluster distinct from other HSDs, particularly those catalyzing dehydrogenations at positions other than the 3β of the steroid nucleus or displaying an opposite stereochemistry for C-3 hydride abstraction. These data are consistent with the suggestion that At3βHSD/D1 and At3βHSD/D2 code two plant 3β-hydroxysteroid dehydrogenase/C-4 decarboxylases isoenzymes.
Plant 3β-Hydroxysteroid Dehydrogenase/Decarboxylase

At3βHSD/D1, and ∆At3βHSD/D2 Containing the At3βHSD/D1 ER Retrieval Signal. Can Complement a Yeast Strain Deficient in Erg26p—To further characterize the function of At3βHSD/D1 and At3βHSD/D2, we performed a yeast complementation assay in the ERG26-deficient strain SDG200 deficient in 3βHSD/D activity (13). In S. cerevisiae, the ERG26 product is an essential enzyme because of the fact that disruption of ERG26 is lethal, and the erg26 strain requires ergosterol or cholesterol supplementation for viability (13). Despite one putative membrane-embedded domain, the lack of the C-terminal ER retrieval signal KKXY in At3βHSD/D2 might result in inefficient localization in the ER yeast C-4 demethylation complex. Thus, we replaced the seven terminal amino acids of At3βHSD/D2 with the KKID sequence to produce ∆At3βHSD/D2 with little documentation in the literature. Additionally, in the case of an enzyme that is part of a membrane-bound multienzymatic complex, interactions with other components of the complex may be necessary for optimum enzymatic activity. Thus, 3βHSD/D activity was assayed in the microsomal extracts and corresponding cytosolic fractions prepared from erg26-pVT-VOID, erg26-pVT-At3βHSD/D1, and erg26-pVT-∆At3βHSD/D2 by using the standard assay uptake for erg26 mutants. The erg26 strain transformed with pVT-At3βHSD/D1 and pVT-∆At3βHSD/D2 was capable of growing aerobically without ergosterol supplementation, whereas erg26/pVT-VOID transformants could grow only on an ergosterol-supplemented medium (supplemental Fig. C). To confirm the authenticity of the complementation by At3βHSD/D1 and ∆At3βHSD/D2, several colonies of the prototrophic strains were picked from the selection plate and grown in an δ-aminolevulinic acid-containing liquid medium devoid of sterol. After sterol extraction, the sterol profiles were analyzed by GC and GC-MS. The strains erg26-pVT-At3βHSD/D1 and erg26-pVT-∆At3βHSD/D2 accumulated more than 70% of C-4-demethylated sterols, including ergosterol (50–52%) as the major sterol, and minor amounts (12–16%) of residual 4,4-dimethylsterols (Table 2). In comparison the auxotropic strain erg26/pVT-VOID grown in a cholesterol-containing media accumulated lanosterol and small amounts of 4α-carboxy-4,14-dimethyl-cholest-8-en-3β-ol (20) as shown previously (13). In the presence of δ-aminolevulinic acid, erg26/pVT-VOID accumulated 4α-carboxy-4β-methyl-cholest-8-en-3β-ol (2) but no ergosterol as shown previously for erg26 (13). These results demonstrate that the A. thaliana At3βHSD/D1 and ∆At3βHSD/D2 cDNAs can efficiently complement the ergosterol auxotroph yeast erg26 strain by restoring growth and endogenous ergosterol synthesis.

At3βHSD/D1 and ∆At3βHSD/D2 Have 3β-Hydroxysteroid Dehydrogenase/C-4 Decarboxylases Activity in Vitro—We next performed an enzymatic assay to test whether the two recombinant Arabidopsis putative 3βHSD/D proteins in the transformed erg26 strain indeed possess 3-hydroxysteroid dehydrogenase/C-4 decarboxylase activity. Overexpression and purification of plant membrane proteins for functional analysis are still relatively unexplored fields for yeast or bacteria with little documentation in the literature. Additionally, in the case of an enzyme that is part of a membrane-bound multienzymatic complex, interactions with other components of the complex may be necessary for optimum enzymatic activity. Thus, 3βHSD/D activity was assayed in the microsomal extracts and corresponding cytosolic fractions prepared from erg26-pVT-VOID, erg26-pVT-At3βHSD/D1, and erg26-pVT-∆At3βHSD/D2 by using the standard assay.
TABLE 2
Sterol composition of mutant erg26 transformed with different plasmids
Values represent the means ± S.D. of the analysis of three to five clones.

| Sterol                     | pVT-VOID | pVT-At3βHSD/D1 | pVT-Δ-At3βHSD/D2 |
|---------------------------|----------|----------------|------------------|
| **Medium supplementation**|          |                |                  |
| Cholesterol               | Yes      | No             | No               |
| β-Aminovulenic-acid       | No       | No             | No               |
| **Sterol composition**    |          |                |                  |
| Zymosterol                | 1.055*   | 10 ± 1.0       | 11 ± 3           |
| Ergosterol                | 1.097    | 50 ± 3         | 52 ± 3           |
| 4α-Methyl-cholesten-24-dien-3β-ol | 1.130 | 12 ± 1         | 13 ± 2           |
| 7β,24(28)-Ergostadienol  | 1.159    | 11 ± 1         | 12 ± 1           |
| Lanosterol                | 1.232    | 86 ± 6         | 81 ± 1           |
| 4,4-Dimethyl-cholesten-24-dien-3β-ol (12) | 1.243 | 8 ± 2       | 5 ± 2            |
| Eburicic                  | 1.276    | 1 ± 0.2        | 1 ± 0.8          |
| 4α-Carbomethoxy-4β,14α-dimethylcholesten-24-dien-3β-ol (20) | 1.484 | 13 ± 6        |                  |

* Values are f_p (GC<sub>1</sub> DB1).

TABLE 3
Apparent kinetic parameters for selected substrates of recombinant Arabidopsis 3βHSD/D1
For each experiment, kinetic parameters of the different substrates were measured in the same microsomal preparation.

| Substrate                          | K<sub>m</sub> (μM) | V<sub>max</sub> (nmol mg⁻¹ h⁻¹) | V/K/V/K<sup>119</sup> |
|------------------------------------|-------------------|-------------------------------|-----------------------|
| 4α-Carboxy-cholesten-7-en-3β-ol    | 134 ± 15<sup>a</sup> | 86 ± 10                       | 1.00                  |
| 3α-Deutero-4α-carboxy-cholesten-7-en-3β-ol (11) | 112 ± 13 | 77 ± 7   | 1.08 ± 0.08         |
| DKIE                               | 383 ± 35          | 5.4 ± 1                       | 0.023 ± 0.001        |
| 4α-Carboxy-4β-methyl-cholesten-24-dien-3β-ol (2) | 274 ± 27 | 113 ± 10 | 0.64 ± 0.02        |
| NAD<sup>b</sup>                   | 8.0 ± 0.5         |                              |                       |

<sup>a</sup> V<sub>max</sub> = k<sub>cat</sub> × [enzyme concentration] (62); thus for two substrates, A and B, using the same enzyme preparation: V<sub>max</sub>/K<sub>m</sub> = V<sub,max</sub>/K<sub>m</sub> = k<sub>cat</sub>/K<sub>m</sub> = k<sub>cat</sub>/K<sub>m</sub>.

<sup>b</sup> Values represent the mean ± S.D. of two independent experiments.

TABLE 4
Apparent kinetic parameters for selected substrates of recombinant Arabidopsis Δ3βHSD/D2
For each experiment, kinetic parameters of the different substrates were measured in the same microsomal preparation.

| Substrate                          | K<sub>m</sub> (μM) | V<sub>max</sub> (nmol mg⁻¹ h⁻¹) | V/K/V/K<sup>119</sup> |
|------------------------------------|-------------------|-------------------------------|-----------------------|
| 4α-Carboxy-cholesten-7-en-3β-ol    | 87 ± 4<sup>a</sup> | 66 ± 1                        | 1.00                  |
| 3α-Deutero-4α-carboxy-cholesten-7-en-3β-ol (11) | 81 ± 5  | 60 ± 2         | 0.97 ± 0.05          |
| DKIE                               | 655 ± 72          | 12 ± 2                        | 0.013 ± 0.001        |
| 4α-Carboxy-4β-methyl-cholesten-24-dien-3β-ol (2) | 204 ± 35 | 45 ± 9   | 0.29 ± 0.01         |
| NAD<sup>b</sup>                   | 3.1 ± 0.4         |                              |                       |

<sup>a</sup> V<sub>max</sub> = k<sub>cat</sub> × [enzyme concentration] (62); thus for two substrates, A and B, using the same enzyme preparation: V<sub>max</sub>/K<sub>m</sub> = V<sub,max</sub>/K<sub>m</sub> = k<sub>cat</sub>/K<sub>m</sub> = k<sub>cat</sub>/K<sub>m</sub>.

<sup>b</sup> Values represent the mean ± S.D. of two independent experiments.

conditions for recombinant 3βHSD/D described under “Experimental Procedures.” The results from these studies revealed that microsomal extracts obtained from erg26-pVT-At3βHSD/D1 and erg26-pVT-Δ-At3βHSD/D2 were able to oxidatively decarboxylate the 3βHSD/D substrate, 4α-carboxycholesten-7-en-3β-ol (1), with a high efficiency in the presence of NAD<sup>+</sup>, to produce a single 3-keto-4-decarboxylated metabolite, cholesten-7-en-3-one (6), which was unequivocally identified by GC-Ms analysis (Table 1 and supplemental Fig. A). 3βHSD/D activity was undetectable in reactions with microsomal extracts of the plant 3βHSD/D with substrate 4α-carboxycholesten-7-en-3β-ol (1) were determined by varying the concentration of (1) by using a constant 50-fold K<sub>m</sub> concentration of NAD<sup>+</sup>. However, the V<sub>max</sub> for NAD<sup>+</sup> could not be measured due to the inability of using saturating concentrations of carboxosterol (1), because it has limited solubility in aqueous media. Under our standard assay conditions, the velocity/substrate concentration curves obey simple Michaelis-Menten kinetics with respect to (1) and NAD<sup>+</sup> cofactor (supplemental Fig. C). The obtained kinetic data for 3βHSD/D1 and -2 are summarized in Tables 3 and 4, respectively.

To gain insight into the enzymatic mechanism of the two 3βHSD/Ds, we compared V<sub>max</sub> and V/K values measured for 3α-protonated 4α-carboxycholesten-7-en-3β-ol (1) with those obtained with its synthetic 3α-deuterated analog (11) in the same microsomal preparation. We used a noncompetitive assay, in which the protonated and deuterated substrates are measured separately. The advantage of the direct comparison method is that it is the only means to determine the primary kinetic deuterium isotope effect on V<sub>max</sub> (δV). For both At3βHSD/D1 and Δ-At3βHSD/D2, the measured primary
deuterium kinetic isotopic effect for (11) was near unity, both for $V$ and $V/K$, within experimental error (Tables 3 and 4 and supplemental Fig. D). Moreover, recovery and GC-MS analysis of the untransformed substrate (11) revealed no loss of deuterium, thus excluding any washout of the deuterium label at C-3 during the incubation procedure.

**Substrate Screening of the At3βHSD/Ds**—We used a series of natural or synthetic substrate analogs to determine the structural requirements of the 3βHSD/Ds. The steroid substrate screen used our standard 3βHSD/D assay conditions (see “Experimental Procedures”) at saturating levels of NAD$^+$ cofactor (50-fold the $K_m$ value). The assay conditions were complemented by analysis of products and residual substrates using GC-MS.

First, a series of 3β-hydroxy-4α-carboxysteroids with distinct nucleus or side chain structures were assayed with 3βHSD/D1. These included (3) and (4), the respective products of the two plant sterol C-4-methyl oxidases (SMO) (4), and thus the most probable substrates for decarboxylation in plants, and (2) the physiological substrate of the yeast 3βHSD/D, which accumulates in the erg26 mutant (13, 32), was also assayed. Because of the difficulty of obtaining sufficient quantities of (3) and (4) that could only be produced enzymatically in vitro, we could not determine the kinetic constants of these two analogs but were able to compare their reaction rates, in the same 3βHSD/D1 enzymatic preparation, with that of the other substrates used at the same concentration. The data indicate that all four 3β-hydroxy-4-carboxysterols (1–4) were dehydrogenated and decarboxylated by At3βHSD/D1 (Fig. 4A and Table 3) and that this isoenzyme did not show a marked preference for any of these four 3β-hydroxy-4-carboxysterols. The corresponding enzymatic 3-oxo-C-4-decarboxylated products of reaction were unequivocally identified as (6–9), respectively, by GC-MS analysis (Table 1).

To probe the stereoselectivity of the plant 3βHSD/D1 for the 3β-hydroxy group of the substrate, the epimer of (1) with the 3α-configuration (5) was synthesized. It was dehydrogenated by the recombinant 3βHSD/D1, but with a 50-fold lower activity, to also produce (6), which was unequivocally identified by GC-MS (Table 3 and Fig. 4A).

Next, we examined the requirement for the C-4-carboxylic substituent. Ursolic acid (15), a triterpene derivative possessing a 3β-hydroxyl group and a distal C-17-carboxyl function, was not metabolized. Converting the carboxyl group in (1) to generate the C-4-carboxymethylated derivative (10), or substitution of the carboxyl group by a methyl group as in the 4,4-dimethylsterols (12) and (13) or the 4α-methylsterol (14), totally abolished the dehydrogenase activity of 3βHSD/D1 in the presence of NAD$^+$ cofactor (Fig. 4A). Indeed, following incubation of cold or radioactive labeled samples of these analogs, TLC and GC-MS analysis of the reaction excluded the formation of the corresponding 3-oxo products. Moreover, by using a series of 3β-hydroxy steroids, pregnenolone (16), trans-androstenone (17), 3β-androstanediol (18), and dehydroepiandrosterone (19), which are known substrates of a number of animal 3βHSDs, we could not detect any oxidative conversion of the 3β-OH by the plant 3βHSD/D1.

Similarly, the structural requirements of Δ3βHSD/D2 were examined in a separate series of experiments by comparing the reaction rates of the substrate analogs in the Δ3βHSD/D2 enzymatic preparation. The data (Fig. 4B and Table 4) indicated that all four 3β-hydroxy-4-carboxysterols (1–4) were dehydroge-
nated and decarboxylated by Δ3βHSD/D2. Δ3βHSD/D2 did not show a marked preference for any of these substrates other than a substantial lower transformation rate observed for (2), the physiological substrate of the yeast 3βHSD/D (which is however not synthesized in plants). The Δ3βHSD/D2 isoenzyme also dehydrogenated (5) the 3α-epimer of (1) at a 50-fold lower rate and was also not able to metabolize compounds (10 and 12–19).

Silencing of Endogenous N. benthamiana 3βHSD/D—In order to learn more about the function of the 3βHSD/D, a cDNA fragment of 500 bp termed Nb3βHSD/D, homologous to the N terminus of At3βHSD/D1 and At3βHSD/D2 (or Δ-At3βHSD/D2), was isolated in N. benthamiana and cloned into the viral TTO vector. This sequence was placed under the control of the tobacco mosaic virus (TMV-U1) coat protein subgenomic promoter in the antisense orientation. Nb3βHSD/D shares 59–57% identity with At3βHSD/D1 and At3βHSD/D2 and thus falls clearly within the 3βHSD/D family (Fig. 3 and supplemental Fig. B).

N. benthamiana plants were inoculated with infectious TTO-Nb3βHSD/D mRNAs (Fig. 5C), and noninfected plants (Fig. 5A) as well as plants infected with the previously described TTO-DXR (Fig. 5B) construct (17) were used as controls. Silencing of Nb3βHSD/D strongly reduced the growth of young leaves (Fig. 5C) compared with uninfected plants and plants infected with TTO-NbDXR (Fig. 5, A and B). To measure the VIGs of 3βHSD/D, semiquantitative RT-PCR analysis was performed using 3βHSD/D primers annealing outside of the region used for the silencing. We observed a 70% reduction of the transcripts in Nb3βHSD/D silenced plants compared with uninfected plants and the TTO-NbDXR control (Fig. 5D).

Approximately 3 weeks after infection, total lipids from young plants, containing sterols and expected carboxysterol derivatives, were extracted and purified by TLC allowing the separation of steryl esters, 4,4-dimethyl-, 4α-methyl-, 4-demethylsterols, and a more polar fraction which was treated with diazomethane to stabilize and decrease the polarity of the carboxysterol derivatives. The sterols were quantified by GC and compared with authentic standards by GC-MS electron impact mass spectra.

Results indicate that the most noteworthy difference between the control and the TTO-Nb3βHSD/D infected plants appeared to be the addition of a novel sterol derivative that represents 2–3% of the total sterol content and is not detectable in extracts from control plants (Fig. 6). This compound was identified as 4α-carboxy-4β,14α-dimethyl-9β,19-cyclo-ergost-24(24'-en)-3β-ol (3) by GC-MS analysis after derivatization and comparison with the MS spectrum of an authentic enzymatically produced sample (4). In addition, a 2-fold accumulation of 4,4-dimethylsterols was observed in the plants infected with TTO-Nb3βHSD/D, whereas the bulk of the sterol profile remained unchanged within the limit of experimental error (data not shown).

**DISCUSSION**

The SDR family includes more than 2000 annotated enzymes that contain a 250–350-amino acid residue core structure, catalyzing NAD(P)(H)-dependent oxidoreductions of great functional diversity (9–11, 33). Despite a sequence identity level of only 15–30% between different SDR members, the three-dimensional folds are quite similar, except for the C-terminal regions. According to the Sanger Institute data base, most of the 3β-hydroxysteroid dehydrogenases show a common 3βHSD architecture (Pfam 01073) found in the present At3βHSD/D isofoms, and a small number (5 of 198) possess an additional C-terminal 200-amino acid residue segment homologous to the reticulon containing two large hydrophobic regions that are likely embedded in the ER membrane (34, 35). Whereas the 3βHSD/D1 cDNA corresponding to the
Plant 3β-Hydroxysteroid Dehydrogenase/Decarboxylase

Control

TTO-Nb3βHSD/D

A

A =
At2g47290 gene encodes a protein that shows the 3βHSD architecture, the 3βHSD/D2 cDNA encodes a protein corresponding to the 3βHSD core domain of the At2g26260 gene product possessing the 3βHSD-reticulon architecture.

The two Arabidopsis clones, as well as the Nicotiana 3βHSD/D, share the typical signatures of the SDR family (supplemental Fig. B), including a nonclassical N-terminal glycine-rich motif TGGGXGXXA, in which the third Gly residue is replaced by Ala. This nonclassical motif is generally found in the SDR group belonging to multifunctional enzyme complexes (11). In addition, the highly conserved YXXK segment (residues 159–163 in 3βHSD/D1), assigned to the catalytic center (36), is found in both At3βHSD/D1, -D2, and Nb3βHSD/D. Therefore, based on the sequence alignment of the plant 3βHSD/Ds with members of the SDR family (Pfam 01073), the conserved residues Ser-131, Tyr-159, and Lys-163 found in At3βHSD/D1, -D2, and Nb3βHSD/D could constitute the consensus catalytic triad, Ser, Tyr, Lys, proposed for SDR catalysis, facilitating the initial oxidation of (1) and analogs and requiring NAD$^+$ as coenzyme (9–10, 36).

The 3βHSD/D proteins show low sequence identity with other members of the SDR family thus forming a differentiated cluster in the phylogenetic tree (Fig. 3). The formation of this distinct subfamily could reflect the remarkable bifunctionality of the 3βHSD/D proteins.

Identification of genes encoding membrane proteins can be achieved using the technique of “functional complementation” in a relevant yeast deletion mutant (37, 38). Complementation of the yeast erg26 mutant by plant 3βHSD/D cDNAs inserted into a vector optimized for yeast expression was expected to restore both growth in the absence of ergosterol and endogenous ergosterol biosynthesis. At3βHSD/D1 and Δ-At3βHSD/D2 restored growth, high levels of ergosterol biosynthesis, and in vitro high catalytically competent 3βHSD/Ds localized exclusively in the corresponding microsomal extracts. In contrast to most SDR proteins, including HSDs, biochemical studies performed in animals (39), yeast (40), and plants (5) have shown that native 3βHSD/D is membrane-bound. Although a single hydrophobic portion was found in the Arabidopsis 3βHSD/D1 and Δ-At3βHSD/D2 proteins, the activities of both recombinant At3βHSD/Ds containing an ER retrieval signal were found in the corresponding microsomal extracts. These data indicate that At3βHSD/D1 and Δ-At3βHSD/D2 are functionally inserted in the yeast C-4-demethylation enzymatic complex and are able to channel endogenous 4α-carboxy-4β-methylcholest-8,24-dien-3β-ol (2) through this complex and the sterol pathway.

Extensive studies performed in yeast have elucidated the protein-protein interactions with regard to sterol biosynthesis and particularly the C-4 demethylation complex (40, 41). Functional association of At3βHSD/Ds with the C-4 demethylation complex could involve a limited number of amino acid interac-

cations, including charged pairing between the partners as shown in a number of membrane-bound electron transfer complexes such as the cytochrome $b_6$, cytochrome $b_5$ reductase (42, 43) or the cytochrome P450-cytochrome P450 reductase (44) complexes. Mutagenesis of the At3βHSD/Ds should be studied to probe which amino acids are necessary for these interactions.

Examination of the apparent kinetic parameters (Tables 3 and 4) of (1) for the At3βHSD/Ds revealed $K_m$ values that are in the same order of magnitude to those of a variety of native post-squalene sterol biosynthetic enzymes from yeast (20) and native (2) or recombinant (45) plant sterol enzymes. In contrast, the apparent rates of At3βHSD/D1 and Δ-At3βHSD/D2 (66 – 86 nmol·mg$^{-1}$·h$^{-1}$), as well as that of the wild type diploid yeast Sc3βHSD/D (366 nmol·mg$^{-1}$·h$^{-1}$; data not shown), appear relatively high in comparison to the rates of other enzymes of the post-squalene sterol pathway measured in the corresponding microsomal preparations (2), including other components of the C-4 demethylation complex such as the SMO from yeast ($V_{m}$ = 1.2 nmol·mg$^{-1}$·h$^{-1}$) (20) or plants ($V_{m}$ = 8.1 nmol·mg$^{-1}$·h$^{-1}$) (4). These data suggest that 3βHSD/D is not rate-limiting for the overall process of C-4 demethylation and that 4-carboxysterols are probably transient intermediates. This is consistent with the observation that they have not been isolated in plant or wild type yeast thus far.

The high catalytic activity of Δ-3βHSD/D2 (Table 4) indicates that the 200-amino acid C-terminal hydrophobic segment (homologous to reticulon present in the complete At2g26260 gene product and absent in Δ-3βHSD/D2) is not involved in 3βHSD/D catalysis. In addition, the calculated molecular mass of 3βHSD/D1 and Δ-3βHSD/D2, 42 kDa for both, is in agreement with the apparent molecular mass of 45 kDa found for the native and catalytically competent 3βHSD/D purified from microsomal extracts of Z. mays (5).

Deuterium kinetic isotope effects have been used extensively to study the mechanism of numerous enzymes and particularly those involving a hydrogen transfer step (for review see Refs. 22, 23, and 46–48). We recently used kinetic isotope effects to study the mechanism of a yeast recombinant plant membrane sterol desaturase (49); however, no such study has been carried out with an HSD thus far. In noncompetitive assays, the primary deuterium kinetic isotope effects for oxidation of 3α-deutero-4α-carboxy-cholest-7-en-3β-ol (11) catalyzed by 3βHSD/D1 and Δ-3βHSD/D2 were near unity, i.e. $D/V = 1.10$. $V/K = 0.93 – 1.03$. Similarly, we did not observe any significant kinetic isotope effect with a microsomal preparation of wild type yeast Sc3βHSD/D (data not shown). This lack of an isotope effect indicates that the 3α-hydrogen–carbon bond cleavage is not the rate-limiting step of the reaction. One possibility to explain the lack of an isotope effect would be that one or several steps of the reaction pathway, including chemical and product release, might be slower than the hydride transfer step and thus completely masked the $D/V$. Another possibility is that (11) behaves as a...
sticky substrate that reacts to give products as fast or faster than it dissociates from the enzyme, i.e. the enzyme-substrate complex has a high commitment to catalysis (47, 48, 50).

Extensive studies using multiple isotope effects have been carried out with a number of enzymes catalyzing oxidative decarboxylations with NADP$^+$ as cosubstrate, exemplified by the malic enzyme (51–53) or the 6-phosphogluconate dehydrogenase (54). In these cases, a stepwise mechanism was shown, and a small isotope effect for the hydride transfer step was measured, comparable with that found with the 3βHSD/Ds studied here. However, measurement of a single isotope effect is not sufficient to elucidate the mechanism of the 3βHSD/Ds, although it would seem logical that 3C–H bond cleavage and decarboxylation are separate events. Determination of the mechanism of the 3βHSD/Ds will require further studies, including in particular the $^{13}$C isotope effect for C–C bond cleavage (53, 54), and variation with pH of primary isotope effects (47, 55–57).

To complete our catalytic analysis, we studied the substrate requirements of each of the two 3βHSD/Ds in a separate series of experiments incubating various substrate analogs with enzymatic preparations. For both 3βHSD/Ds isoenzymes, the data showed a substrate specificity that required free 3β-hydroxyl and C-4 carboxyl groups indicating that both recombinant At3βHSD/Ds are highly specific for 3β-hydroxy-4α-carboxysterols. In contrast, substrate requirements were quite tolerant in terms of variations of the sterol nucleus and side chain structures. Remarkably, the data did not reveal for either of the two 3βHSD/Ds a clear preference between substrates possessing one or two substituents at C-4. In addition, the substrate preferences of the two 3βHSD/Ds were found to be very similar. This is in contrast with the strict and distinct structural requirements observed for the two distinct SMOs metabolizing 4,4-dimethyl- and 4α-methylsterols, respectively, to produce the corresponding carboxysterols that are the physiological substrates of the present 3βHSD/Ds (4). Furthermore, (2), the typical substrate of the yeast 3βHSD/D, was efficiently oxidized by both At3βHSD/Ds, whereas 4,4-dimethyl-zymosterol (12), the substrate of the yeast SMO, was not metabolized by any of the plant SMOs (20). These data suggest that a single type of 3βHSD/D is metabolizing the products of the two distinct SMOs inside the plant C-4 demethylation complex.

The VIGS experiments carried out in this study led to young N. benthamiana plants exhibiting a clear biochemical phenotype. As this biochemical phenotype was observed in several distinct infected plants but not in plants infected with the TTO-DXR construct or noninfected plants, it can clearly be ascribed to the presence of the Nb3βHSD/D cDNA. These biochemical changes observed after infection with TTO-Nb3βHSD/D, correlating with the specific reductions in Nb3βHSD/D mRNA levels, confirm the genetic silencing of the corresponding Nb3βHSD/D endogene.

Silencing of Nb3βHSD/D resulted in a substantial accumulation of a novel 4α-carboxysterol, 4α-carboxy-4β,14α-dimethyl-9β,19-cyclo-ergost-24(24$^\alpha$)-en-3β-ol (3), involved in the pathway for removing the first methyl group at C-4, thereby confirming in planta the function of the present 3βHSD/Ds. Recent studies in A. thaliana indicate that plants compromised early in the sterol biosynthetic pathway, i.e. upstream of the removal of the second C-4 methyl group, such as the fackel mutant defective in C-14 reductase (58, 59), show severe defects in development and embryogenesis, whereas those affected later in the pathway, such as dwp7/ste1 (60) or dwp1/ DIM (61), do not. Thus, in the event of an impaired expression of a gene upstream of the second C-4 demethylation step, plants might develop regulatory processes to reduce these defects, thereby limiting as in the present case the accumulation of (3). In these lines, a limited accumulation of the substrates of the SMOs in SMO-silenced plants was also observed previously by using the same VIGS approach in N. benthamiana (8). In this respect, 3βHSD/D-silenced plants, albeit accumulating modest amounts of (3), present substantial developmental alterations that will need further work to be precisely characterized.

The present combination of molecular and biological chemical approaches allowed a thorough identification and functional characterization of 3βHSD/D as one further step in the completion of the molecular inventory of sterols synthesis in higher plants. Genetic redundancy or lethality may account for the failure to isolate plant 3βHSD/D genes by screening of phenotypes by using a genetic approach. Given that plant mutants affected in 3βHSD/D have not been reported thus far, the present study provides important clues for the physiological roles of C-4-substituted sterols in photosynthetic eukaryotes. Finally, these two novel oxidative decarboxylases constitute the first plant hydroxysteroid dehydrogenase genes of the SDR superfamily to be molecularly and enzymatically characterized.

Acknowledgments.—We are indebted to Annie Hoeft for GC-MS analysis and helpful technical assistance. We thank Monto Kumagai and Large Scale Biology Corp. for the TT0 vector. We are grateful to J. D. Sauer for recording the NMR spectra.

REFERENCES

1. Benveniste, P. (2004) Annu. Rev. Plant Physiol. Plant Mol. Biol. 55, 429–457
2. Bouvier, F., Rahier, A., and Camara, B. (2005) Prog. Lipid Res. 44, 357–429
3. Lees, N. D., Skaggs, B., Kirsch, D. R., and Bard, M. (1995) Lipids 30, 221–226
4. Pascal, S., Taton, M., and Rahier, A. (1993) J. Biol. Chem. 268, 11639–11654
5. Rondet, S., Taton, M., and Rahier, A. (1999) Arch. Biochem. Biophys. 366, 249–260
6. Pascal, S., Taton, M., and Rahier, A. (1994) Arch. Biochem. Biophys. 312, 260–271
7. Bard, M., Bruner, P. A., Pierson, C. A., Lees, N. D., Biermann, B., Frye, L., Koegel, C., and Barbuch, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 186–190
8. Darnet, S., and Rahier, A. (2004) Biochem. J. 378, 889–898
9. Oppermann, U., Filling, C., and Jörnwall, H. (2001) Chem. Biol. Interact. 130–132, 699–705
10. Oppermann, U., Filling, C., Hult, M., Shafqat, N., Wu, X., Kindh, M., Shafqay, E., Nordling, E., Kallberg, Y., Persson, B., and Jörnwall, H. (2003) Chem. Biol. Interact. 143, 247–253
11. Kallberg, Y., Oppermann, U., Jörnwall, H., and Persson, B. (2002) Protein Sci. 11, 636–641
12. Hyndman, D., Bauman, D. R., Heredia, V. V., and Penning, M. (2003) Chem. Biol. Interact. 143, 621–631
13. Gachotte, D., Barbuch, R., Gayl, J., Nickels, E., and Bard, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13794–13799
