5alpha-Reductase Activity in Lycopersicon Esculentum: Cloning and Functional Characterization of Ledet2 and Evidences of the Presence

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5α-Reductase activity in *Lycopersicon esculentum*: Cloning and functional characterization of *LeDET2* and evidence of the presence of two isoenzymes

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Abstract

The full-length cDNA (*LeDET2*) encoding a 257 amino acid protein homolog of *Arabidopsis* DET2 (AtDET2) was isolated in tomato (*Lycopersicon esculentum)*. LeDET2 has 76% similarity with AtDET2 and structural characteristics conserved among plant and mammalian steroid 5α-reductases (5αRs). *LeDET2* is ubiquitously expressed in tomato tissues with higher levels in leaf than in stem, root, seed and callus. When expressed in mammalian cells (COS-7), recombinant LeDET2 was active on substrates typical of mammalian 5αRs (progesterone, testosterone, androstenedione), but reduced at very low levels campestenone, the substrate described for AtDET2. Similar results were obtained with the expression in COS-7 of recombinant AtDET2 that showed 5αR activity for progesterone and not for campestenone. Recombinant LeDET2 was inhibited by several inhibitors of the human 5αRs and the application of an active inhibitor to tomato seedlings induced dwarfism and morphological changes similar to BR-deficient mutants. In tomato tissues, campestenone was 5α-reduced in leaf, stem and root homogenates, like progesterone and testosterone, while androstenedione was converted to testosterone, evidencing for the first time a 17β-hydroxysteroid dehydrogenase activity in plants. Moreover, two separate 5αR activities with different kinetic characteristic and response to inhibitors were characterized in tomato tissues. The presence of two 5αR isoenzymes was demonstrated also in *Arabidopsis* using the *det2-1* mutant, in which a residual 5αR activity for campestenone and progesterone was evidenced and characterized. Therefore, the existence of two isoenzymes of 5αR is probably characteristic of the whole plant kingdom highlighting the similarities between the animal and plant steroid biosynthetic pathways.

Keywords: 5α-Reductase; *DET2*; *Lycopersicon esculentum*; Brassinosteroids; Steroid hormones; Inhibitors

1. Introduction

Brassinosteroids (BRs) are steroidal hormones essential for normal growth and development of plants. They are C_{27}, C_{28} and C_{29} steroids depending on the alkyl-substitution pattern of the side chain. Among them, brassinolide is the most biologically active molecule and, together with its C_{28} congeners, it is widely distributed through the plant kingdom [1,2]. Application of BRs induces a broad spectrum of responses, including effects on elongation, cell division, germination, senescence, reproductive and vascular development, and enhancement of stress tolerance [for a review, see ref. [3]]. Due to all these effects on plant growth and development, BRs are considered the plant homologs of steroid hormones in the animal kingdom. Moreover, surprising similarities exist between the BRs and animal steroids hormones biosynthetic pathways.

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The reduction of the $\Delta^{4,5}$ double bond via a 5α-reductase is a key step in the biosynthesis of BRs since all known biologically active BRs lack double bonds in the A and B rings and have a 5α-reduced stereochemistry. Support to this assumption comes from the examination of the phenotype of the *Arabidopsis* BR-deficient mutant *det2-1* that is defective in the conversion of (24*R*)-24-methylcholest-4-en-3-one (campestenone) to (24*R*)-24-methyl-5α-cholestan-3-one (5α-campestanone) [4]. *Arabidopsis det2-1* mutants are dwarf and de-etiolated plants in which dark-grown seedlings exhibit short hypocotyl and open cotyledons characteristic of light-grown plants. This phenotype can be reverted by application of brassinolide suggesting that *Arabidopsis DET2* (*AtDET2*) is involved in the biosynthesis of BRs. *AtDET2* is the first gene isolated in plants coding for a steroid 5α-reductase. AtDET2 shows considerable similarity with mammalian steroid 5α-reductases [5].

Recombinant AtDET2 expressed in human embryonic kidney 293 cells was able to reduce several 3-oxo, $\Delta^{4,5}$ mammalian steroids, including progesterone, testosterone and androstenedione. Moreover, the *Agrobacterium*-mediated transformation of *det2-1* mutants with human 5α-reductases rescued the mutant phenotype [6]. These observations indicate that AtDET2 is the functional homolog of the mammalian steroid 5α-reductases confirming the existence of a conserved steroid metabolic pathway between plants and animals.

The human 5α-reductase is a system of two isoenzymes, named 5α-reductase-1 and 5α-reductase-2, with different chromosomal localization and tissue distribution. The two isoforms have diverse enzyme kinetic parameters, pH optima and response to inhibitors [7]. The main substrate is testosterone that is reduced to the more active androgen dihydrotestosterone, and the two human isoenzymes have different roles in many androgen dependent physiological and pathological processes. Beside testosterone, many others 3-oxo, $\Delta^{4,5}$ steroids (androstenedione, progesterone) are 5α-reduced by the two human 5αRs. Recently the existence of two 5αR isoenzymes was demonstrated in *Solanum malacoxylon*, with differences between the two isoforms resembling those described in humans. The two 5αRs of *Solanum* have in fact different kinetic characteristics, pH optima, and a differential response to several inhibitors of the human system [8]. These data lengthen the similarities between human and plant steroid metabolism.

In order to extend the characterization of the 5α-reductase system in plants we have chosen tomato (*Lycopersicon esculentum*) as an important horticultural crop and an excellent model system for biochemical and genetic analysis of plant growth and development.

In this paper, we report the molecular cloning and the functional characterization of *LeDET2*, the homolog in *L. esculentum* of *Arabidopsis thaliana* steroid 5α-reductase *AtDET2*. Moreover, we give biochemical evidences of the presence of more than one enzyme with 5α-reductase activity in *L. esculentum* and *A. thaliana*. These results, together with those obtained in *S. malacoxylon*, suggest that the existence of two isoforms of this enzyme is a general feature of the whole plant kingdom and highlights the similarities with the mammalian 5αR system.

2. Materials and methods

2.1. Plant materials, growth conditions, and inhibitor treatments

Lycopersicon esculentum seeds (cv Saint Pierre) were used in this study. Seedlings were grown in the greenhouse for 2–12 weeks to compare aerial organs. Alternatively, the seeds were surface-sterilised by wrapping seeds in cheesecloth and immersing them sequentially in 70% ethanol for 1–2 min, and then in 1.6% sodium hypochlorite for 10 min. Seeds were rinsed four to five times with sterile distilled water. The seeds were germinated in dark in flasks containing one-half-strength Murashige and Skoog (MS; Duchefa, Haarlem, the Netherland) liquid medium [9] supplemented with 1% sugar. Radicles, hypocotyls and cotyledons were sampled from seedlings after 2 weeks of liquid culture, and 1 week in the case of the whole seed. Similarly, leaf, stem and roots were sampled from plants after 12 weeks of growth in the greenhouse. All samples were frozen in liquid nitrogen and stored at −80 °C until used for RNA extraction or for 5α-reductase activity assay.

Calli of tomato were established from sterile cotyledon on solid MS medium supplemented with 2 mg L^{-1} 2,4-D 2 mg L^{-1} IAA and 0.3 mg L^{-1} kinetine (Duchefa), subcultured monthly and maintained in growth chamber at 23 °C under fluorescent light (photon flux density of 90 μE m^{-2} s^{-1} from Osram daylight) with a photoperiod of 16 h light and 8 h dark. Samples were taken from cultures after 10 days from subculture, they were frozen in liquid nitrogen and stored at −80 °C until used for RNA extraction.

For the inhibition experiments, after 1 weeks of liquid culture, seedlings were transferred in Magenta vessel (9 per vessel) containing solid MS with the addition of increasing concentration of AFA76, ranging from 8 to 20 μM, dissolved in absolute ethanol (0.2% final concentration in the medium). AFA76 was synthesized in our laboratories according to the described methods [10]. Control seedlings were grown on MS medium supplemented with ethanol only. Plants were grown at 23 °C under fluorescent light (photon flux density of 180 μE m^{-2} s^{-1} from Osram daylight) with a photoperiod of 16 h light and 8 h dark in growth chamber. After 10, 20 and 30 days of culture the hypocotyl lengths were measured.

Seeds of wild-type *A. thaliana* (ecotype Col-0) were purchased from LEHLE Seeds (Round Rock, TX, USA). Seeds of *Arabidopsis det2-1* mutant [11] were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham University, UK, stock no. N6159, ecotype Columbia).

Seeds of *Arabidopsis* wild type and *det2-1* mutants were sown on MS solid medium containing 1% sucrose

in Magenta vessels. They were incubated in growth chamber at 23 °C under fluorescent light (photon flux density of 180 μE m^{-2} s^{-1} from Osram daylight) with a photoperiod of 16 h light and 8 h dark.

2.2. Cloning of LeDET2 and AtDET2

The search for putative steroid 5α-reductase in *L. esculentum* was performed using the *A. thaliana* steroid 5α-reductase *DET2* nucleotide sequence as a query in the GenBank database with the BLASTn program [12]. 3′RACE experiments were performed on 4 μg total RNA prepared from leaves of in vitro tomato plants using the RLM-RACE kit (GeneRacer; Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Gene-specific primers used in 3′RACE were T-RACE1 and nested T-RACE2 (Table 1). The RACE products were cloned into pCR4Blunt-TOPO (Invitrogen) and sequenced.

Full-length *LeDET2* cDNA was obtained by RT-PCR performed on total RNA derived from leaves of in vitro tomato seedlings using the forward primer Tom-L2 and the reverse primer Tom-R5 (Table 1). Full-length *LeDET2* cDNA was cloned in the mammalian expression vector pTarget (Promega, Madison, WI, USA), sequenced and designated pT-TOM. As a control, a truncated sequence of *LeDET2* was also expressed in the pTarget vector (Mock). This construct lacks of the first 42 nucleotides containing the starting codon ATG for translation.

Full-length *AtDET2* cDNA was obtained by RT-PCR performed on total RNA derived from leaves of in vitro *Arabidopsis* seedlings using the forward primer Det-L3 and the reverse primer Det-R3 (Table 1). The amplified cDNA was ligated into the expression vector pTarget and sequenced.

Escherichia coli TOP10 (Invitrogen) and JM109 (Promega) were used as hosts for plasmid amplifications.

2.3. PCR analysis of genomic DNA

Genomic DNA was extracted from 2 g of tissue, according to Chen and Dellaporta [13]. For PCR analysis, genomic DNA was amplified with different sets of primers: Tom-L2 and Tom-R2; Tom-L2 and Tom-R3; Tom-L2 and Tom-R5; Tom-L3 and Tom-R2; Tom-L3 and Tom-R3; Tom-L3 and Tom-R5. The primers Tom-L2, Tom-L3, Tom-R2, Tom-R3 and Tom-R5 correspond to position 42, 381, 703, 626 and 869, respectively on *LeDET2* cDNA (Table 1). The amplification product obtained with Tom-L2 and Tom-R5 on genomic DNA was sequenced.

2.4. DNA sequence analysis

DNA sequences were determined at MWG-Biotech Company (Germany) or/and by automated sequencing using an ABI Prism 310 Genetic Analyzer following the BigDye Terminator chemistry (Applied Biosystems, Foster City, CA, USA). All sequences were determined in both senses of the DNA.

The ClustalW program (version 1.8) on the server (http://www.ebi.ac.uk/clustalw/) was used to align both the amino acid and the nucleotide sequences. The aligned amino acid sequences were shaded using the program Boxshade, available on the server at the European Molecular Biology Network (http://www.ch.embnet.org/software/BOX_form.html). Prediction of transmembrane helices was made with MEMSAT (http://bioinf.cs.ucl.ac.uk/psipred/).

Phylogenetic and molecular evolutionary analyses were conducted on the amino acid sequences using *MEGA* version 2.1 [14]. For statistical analysis, 300 bootstrap replications [15] were analysed.

2.5. Cell culture, transient transfections, 5αR activity assay

The COS-7 cell line (derived from African green monkey kidney cells) was used in this study for transient transfection experiments. COS-7 cells were maintained in Dulbecco's minimal essential medium (DMEM; Sigma, San Luis, CA, USA) supplemented with 5% fetal calf serum (FBS; Sigma), 100 mg mL^{-1} streptomycin (Sigma), and 100 U mL^{-1} penicillin (Sigma). Cells were grown at 37 °C in an atmosphere of 95% air and 5% CO_2. They were subcultured as required. COS-7 cells were transfected by electroporation with the

Table 1
Primers used for cDNA isolation, genomic DNA amplifications and RQ RT-PCR experiments

Primer name	Sense/antisense	Nucleotide sequence
T-RACE1	Sense	5′-CACAAAGAACAATTTCCCGCTGAATA-3′
T-RACE2	Sense	5′-GATACCGAGAGGTGGGCTTTTTGAT-3′
Tom-L2	Sense	5′-GCAAAATTAGCTAGCAGACACCA-3′
Tom-R5	Antisense	5′-TGCATTTAAATCAACAATACGATACA-3′
Tom-L3	Sense	5′-AAAGAACAATTTCCCGCTGA-3′
Tom-R2	Antisense	5′-CCCAAGACCAGGTCATCAAA-3′
Tom-R3	Antisense	5′-AAAAGCCCACCTCTCGGTAT-3′
Det-L3	Sense	5′-TTCCATAACCCGAAAAATGG-3′
Det-R3	Antisense	5′-CACAGTTTGTTGTGCATTGTTG-3′
Tomtub2up	Sense	5′-CATTCAAGGAGGTCAATGTG-3′
Tomtub1dw	Antisense	5′-GCATCATTCTATCTGGGTAC-3′

constructs previously described. Briefly, cells maintained in monolayer culture in the exponential growth phase were detached by a 0.04% trypsin solution (Sigma) and suspended in hypoosmolar electroporation buffer (Eppendorf AG, Hamburg, Germany) at the concentration of 1.5×10^6 cells mL^{-1}. This suspension was gently mixed with plasmid DNA at the final concentration of 5 $\mu g\,mL^{-1}$ and transferred into electroporation cuvettes (Eppendorf AG). Cells were subjected to one pulse of 600 V for 40 μs using the Multiporator electroporation system (Eppendorf AG). After the pulse, cells were maintained in the cuvette for 10 min at room temperature and transferred in 100 mm culture dishes with DMEM medium supplemented with 10% fetal calf serum, 100 $mg\,mL^{-1}$ streptomycin, and 100 $U\,mL^{-1}$ penicillin. Forty-eight hours after transfection 5α-reductase activity was assayed in cell lysates as described [8] with campestenone, progesterone, testosterone and androstenedione as substrates. The radioactive substrates [7-^{3}H(N)] androst-4-ene-3,17-dione (24 Ci $mmol^{-1}$), [1,2,6,7-^{3}H(N)] progesterone (97 Ci $mmol^{-1}$), [1,2,6,7-^{3}H(N)] testosterone (80 Ci $mmol^{-1}$) was purchased from NEN-Life Science Products Inc. (Boston, MA, USA). Unlabelled steroids (progesterone, testosterone, androstenedione), were purchased from Sigma. Campestenone and 5α-campestanone were prepared as described [8]. (24*R*)-24-methyl-[2,4,6(n)-^{3}H]cholest-4-en-3-one (67 Ci $mmol^{-1}$) was purchased from Amersham Pharmacia Biotech.

2.6. RNA isolation and RT-PCR analysis

Total RNA was isolated using the RNEasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase I (Qiagen) during RNA purification following the manufacturer's protocol. The amount of total RNA was determined by UV spectrophotometry.

Tissue-specific expression of *LeDET2* was analysed in leaves, roots and stems from 12-week-old plants, 2-week-seedlings and from callus. Relative quantitative RT-PCR (RQ RT-PCR) was performed starting from 2 μg of total RNA. The RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's instructions with Oligo$(dT)_{14}$. Control PCR was run to check the absence of genomic DNA. One microliter of the reverse-transcribed sample, in which tubuline gene used as constitutive control was most abundant, was PCR amplified using the tubuline primers Tomtub2up and Tomtub1dw (Table 1). The cycle conditions were 94 °C for 5 min followed by 16–40 cycles at 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min. The PCR products were agarose gel electrophoresed, blotted, hybridised with specific tubuline-labelled probe and quantified using Kodak 1D Image Analysis Software v. 3.5.4 (Scientific Imaging Systems Eastman Kodak Company, Rochester NY 14650, USA). A cycle number, which corresponded to the linear phase of the PCR reaction, was identified and used in a subsequent PCR of all samples to identify variations in product yield. The appropriate volume needed to standardise each sample relative to that of the reference sample was determined empirically and used in all following PCR reactions. In the same way, we identified *LeDET2* cycle number that corresponded to the linear phase of PCR reaction, using the standardised volume for each sample. The primers used were: Tom-L2 and Tom-R2 (Table 1), which correspond to position 42 and 704 of the *LeDET2* cDNA sequence.

The thermal cycling conditions were 94 °C for 5 min, followed by 27 cycles for tubuline and 32 cycles for *LeDET2* using Taq Polymerase System (Invitrogen).

2.7. 5αR activity assay on tissue homogenates

Freshly harvested leaf, stem, root tissues of soil-grown tomato plants and in vitro grown *Arabidopsis det2-1* seedlings were frozen in liquid nitrogen and stored at −80 °C. Vegetal homogenates were prepared and assayed for 5αR activity as described [8] with campestenone, progesterone, testosterone and androstenedione as substrates.

2.8. Determination of kinetic constants

Saturation curves were determined using a concentration range 0.01–10 μM of campestenone and 0.01–100 μM of progesterone, testosterone, and androstenedione.

Velocities were plotted against substrate concentrations and the apparent K_m and V_{max} values were calculated using a non-linear regression procedure based on Michaelis–Menten equation. An Eadie–Hofstee plot of velocity over substrate concentration was also used, as this plot is reportedly best suited to detect isoenzyme activities [16]. The analysis of data was performed with the computer program Grafit 4.0.16 (Erithacus Software, Horley, Surrey, UK).

2.9. Inhibition test on cell lysates

The following inhibitors of human 5αR system, showed in Fig. 4, were used in this study: steroid inhibitors (4-MA, VG106, DSMEN21, PD91, PD17, Finasteride) and non-steroid inhibitors (AS601811, AFA27, AFA76, AFA131, AFA192). Finasteride was a kind gift of Merck Sharp & Dohme Research Laboratories. 4-MA was synthesized in our laboratories according to the described methodologies [17]. The 10-azasteroids VG106, DSMEN21, PD91, PD17 were synthesized as described [18,19]. The non-steroid inhibitors AS601811, AFA27, AFA76, AFA131, AFA192 were synthesised as reported [10,20,21].

Stock solutions of the inhibitors were prepared at a concentration of 1 $mg\,mL^{-1}$ in ethanol. These solutions were stable for 1 year from the preparation. Working solutions at concentrations below 1 $mg\,mL^{-1}$ were freshly prepared in ethanol. The concentration of inhibitor was 10 μM. The percentage of conversion of the inhibitor was normalized to the control (percent of conversion without the inhibitor) and results were reported as residual 5α-reductase activity.

3. Results

3.1. Molecular cloning of LeDET2 from tomato

Search in GenBank for *L. esculentum* putative steroid 5α-reductase reported the tomato EST BG124294 (GenBank/EMBL) with considerable similarity to the *A. thaliana* steroid 5α-reductase *AtDET2*. This EST sequence terminated upstream of the predicted translational terminator codon. Specific primers were designed in order to isolate the remaining 3′ end by 3′-rapid amplification of cDNA ends (3′RACE) (Table 1). 3′RACE was performed on total RNA extracted from leaves of in vitro seedlings. Full-length cDNA (986-bp) of a putative 5α-reductase gene from tomato was obtained. The full-length clone contained 49 bp of 5′-untranslated sequence, an ORF of 774 bp and 163 bp of 3′-untranslated sequence. The nucleotide sequence showed 53% identity to the *AtDET2* cDNA.

The absence of introns in the ORF sequence was demonstrated by PCR analysis and sequencing of PCR products. PCR amplifications were performed on genomic DNA with the following sets of primers: Tom-L2 and Tom-R2; Tom-L2 and Tom-R3; Tom-L2 and Tom-R5; Tom-L3 and Tom-R2; Tom-L3 and Tom-R3; Tom-L3 and Tom-R5 (Table 1). The amplifications with all sets of primers resulted in one band of 662, 584, 828, 322, 246, 490, respectively (data not shown). The sizes of the amplification products were the same of those obtained by PCR amplifications performed on cDNA with these sets of primers, suggesting the lack of introns in the region between the primers. The amplification product obtained with Tom-L2 and Tom-R5, containing the complete ORF, was sequenced. The results demonstrated that the sequence amplified corresponded to that of cDNA, confirming the lack of introns in the coding region of the isolated gene.

The complete open reading frame cDNA sequence encodes a polypeptide of 257 amino acid residues. The deduced amino acid sequence has 56% identity with AtDET2 and the sequence similarity increases to 76% when conservative substitution is taken into account (Fig. 1). It also has sequence similarity to members of mammalian steroid 5α-reductases (about 35% sequence identity). In support of a function as steroid 5α-reductase, the Glu-199 residue of tomato protein aligned with an invariant Glu residue of mammalian enzymes that is absolutely required for activity. This residue is also conserved in AtDET2 but is replaced by Lys-204 in the mutant det2-1 protein. Furthermore, the Gly-35 residue matched the conserved Gly-34 residue of human 5αReductase-2 isoenzyme implicated in testosterone binding. In addition, the sequence contains six (Arg-148, Pro-183, Gly-185, Asn-195, Gly-198, and Arg-249) of seven conserved amino acids that are part of a cofactor-binding domain typical for mammalian 5α-reductases [7].

The *L. esculentum* amino acid sequence contains six potential transmemembrane-spanning domains according to the program MEMSAT as well as AtDET2 predicted by the same program.

These observations indicated that this gene is the tomato *DET2* homolog and was named *LeDET2* (*L. esculentum DET2*, accession no. AJ786362).

A phylogenetic relationship of 5αR amino acid sequences (Fig. 2) indicates that LeDET2 was in the same branch as did soybean, cotton and AtDET2. 5αR of rice, the only monocot sequence available, was located in a separate branch. The mammalian 5αRs were clustered in separate clades as expected.

3.2. LeDET2 expression in tomato tissues

Organ specific expression of *LeDET2* was analysed by quantitative RT-PCR relative to the tubuline RT-PCR product. Transcripts were analysed in adult and seedling tissues, whole seed after 1 week of imbibition and callus established from leaf.

For each type of tissue four plant samples were analysed and the averages of transcript abundance are shown in Fig. 3. In soil-grown plants, the expression of *LeDET2* was greater in leaf than in stem and root. No considerable differences in the expression were found among seedling tissues and callus. In germinating seed, *LeDET2* was expressed in amount similar to stem and root of soil-grown plants.

3.3. Functional analysis of tomato LeDET2 and Arabidopsis DET2 expressed in COS-7 cells

To investigate whether *LeDET2* encodes a functional steroid 5α-reductase, the gene was functionally expressed in COS-7 (monkey, kidney) mammalian cell line. The coding region of *LeDET2* gene, obtained by PCR amplification with Tom-L2 and Tom-R5 primers, was cloned into the mammalian expression vector pTarget and the resulting plasmid, designated pT-*LeDET2*, was introduced into COS-7 cells by electroporation transfection method.

Forty-eight hours after transfection RT-PCR analysis was performed to verify the expression of the *LeDET2* gene in transfected cells and cell lysates were assayed for 5α-reductase activity. The following tritium-labelled steroidal substrates were used: [^{3}H]campestenone, the substrate of AtDET2 and [^{3}H]progesterone, [^{3}H]testosterone, and [^{3}H]androstenedione, substrates typical of mammalian 5αRs. The 5α-reductase activity was assayed in non-transfected cells and in cells transfected with the pTarget vector containing a truncated sequence of *LeDET2* (Mock) as control. Mock-transfected and non-transfected COS-7 cell lysates showed comparable and very low levels of endogenous 5α-reductase activity with all substrates tested (data not shown). The specific 5αR activity was then calculated subtracting the background of non-transfected cells. The formation of 5α-reduced progesterone was linear from 10 to 100 μg of protein and over a 60 min period of incubation, while the formation of 5α-reduced androstenedione and testosterone

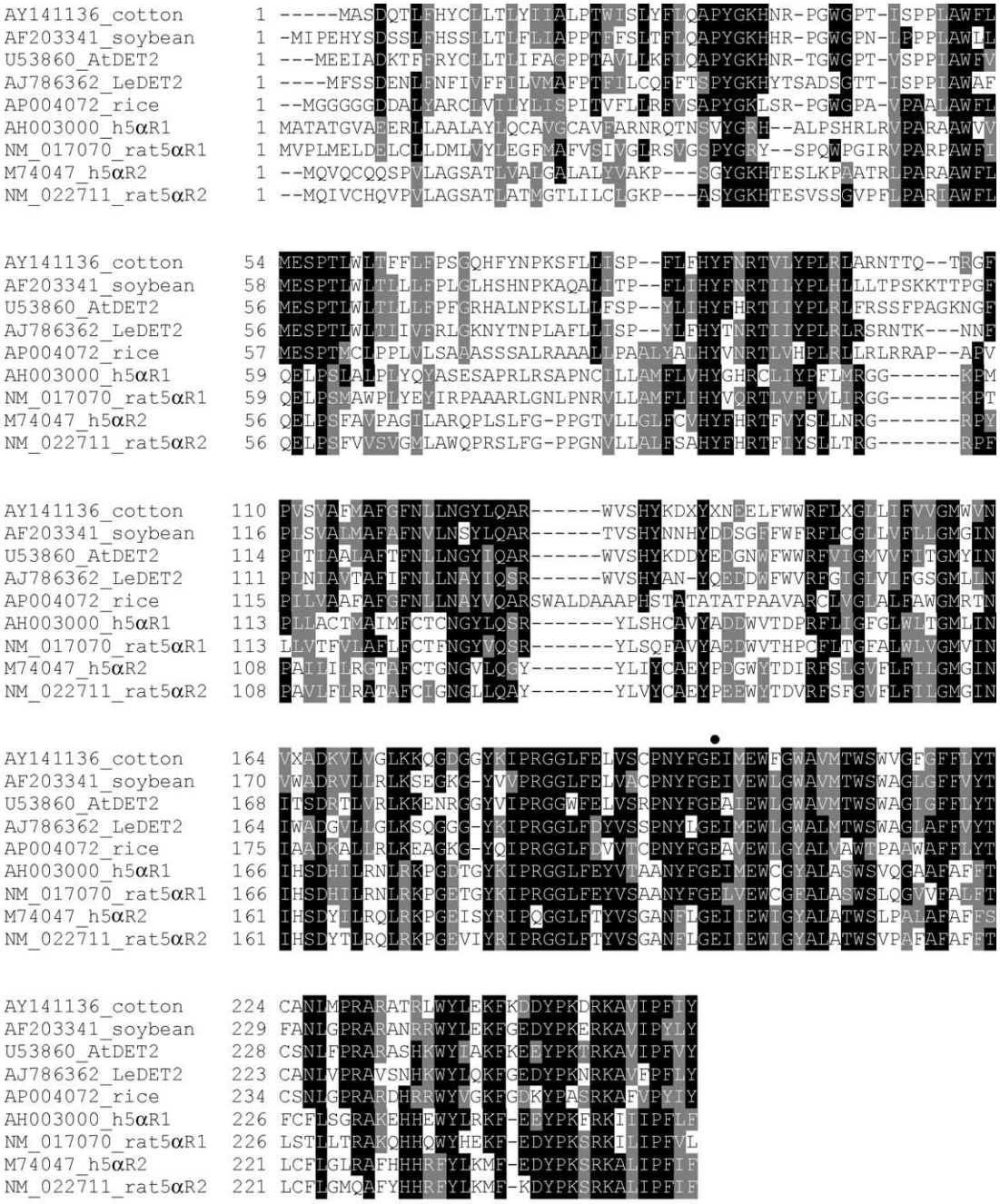

Fig. 1. Multiple sequence alignment of LeDET2 with other steroid 5α-reductases. ClustalW version 1.8 was used to align the amino acid sequence of LeDET2 with *Arabidopsis* DET2, three putative plant DET2 homologous proteins, and mammalian steroid 5αRs. Black and grey shades indicate identical and similar amino acid residues, respectively. The highly conserved Glu residue, which is altered in the *Arabidopsis det2-1* mutant and corresponds to Glu-199 of LeDET2, is marked by a dot.

was linear from 50 to 200 μg of protein and over a 120 min period of incubation (data not shown).

Fig. 4 shows the 5α-reductase activity determined in transfected cells with the substrates at the concentration of 50 nM. The highest 5α-reductase activity was obtained with progesterone, while campestenone was reduced at very low levels.

Table 2 shows the apparent K_m and V_{max} values determined for LeDET2 protein expressed in COS-7 cells using progesterone, testosterone and androstenedione as substrates. The kinetic parameters for campestenone were not determined because of the low affinity of this molecule for the enzyme. Total proteins (20–100 μg) extracted from transfected and non-transfected cells were incubated with tritium-labelled substrates in the presence of increasing concentrations of substrate (0.01–100 μM) and 1 mM NADPH as cofactor. The kinetic constants were calculated

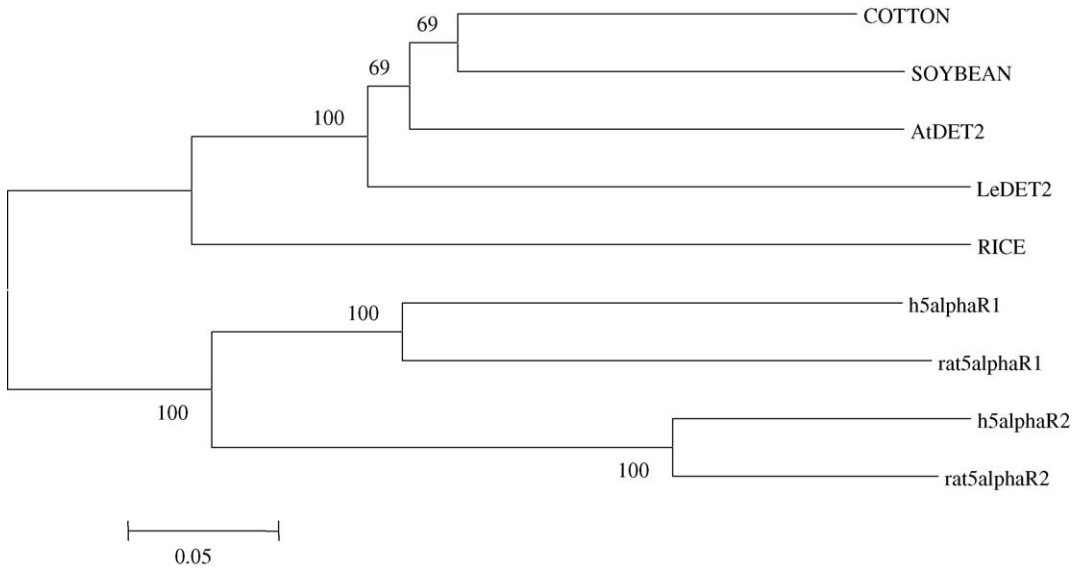

Fig. 2. Phylogenetic relationship among LeDET2 and other steroid 5αRs. Unrooted neighbor-joining analysis using the MEGA2 program for LeDET2, AtDET2, soybean, cotton, rice and mammalian (human and rat) 5αR amino acid sequences. The numbers refer to the boostrap values.

using the non-linear regression procedure based on the Michaelis–Menten equation. The recombinant LeDET2 protein had the highest affinity for progesterone with an apparent K_m value of 0.75 μM, while the apparent K_m values for androstenedione and testosterone were 26 and 40 μM respectively.

Similarly, AtDET2 expressed in COS-7 cells with the same method used for LeDET2 displayed no measurable 5α-reductase activity for campestenone, while progesterone was reduced to 5α-dihydroprogesterone at high levels (data not shown). The kinetic constants of AtDET2 expressed in

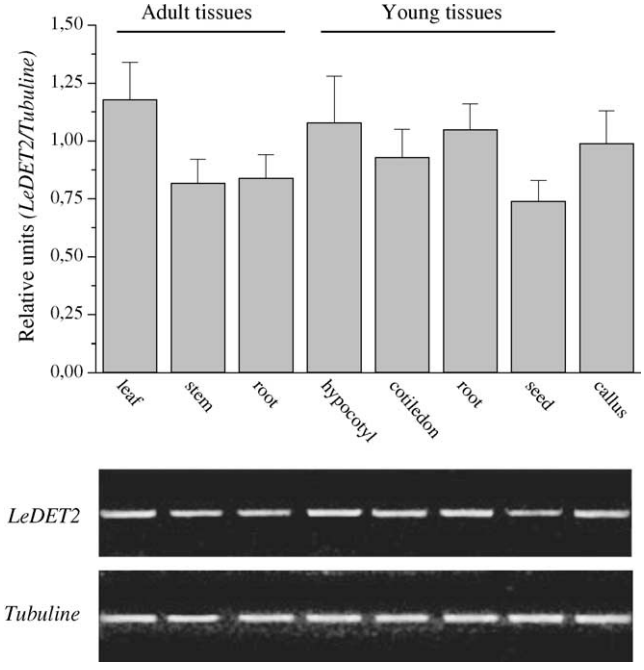

Fig. 3. Expression analysis of *LeDET2* in tomato tissues. Quantitative RT-PCR estimation of specific *LeDET2* transcripts relative to the tubuline in different tissues from 12-week-old plants, 2-week-old seedlings and from callus. Signal values obtained from *LeDET2* were normalised with the tubuline signal value, and the resulting mean values were presented as relative units. Error bars represent S.D.

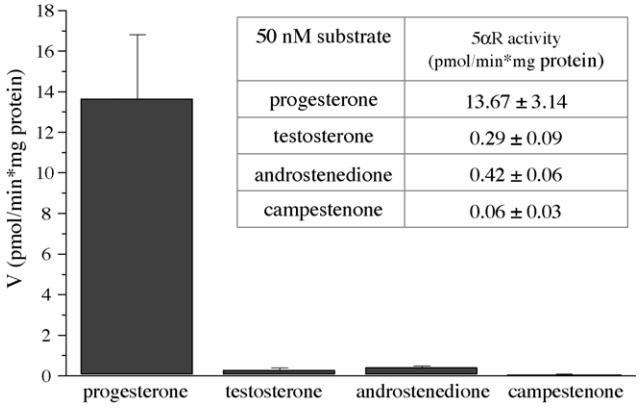

Fig. 4. Functional analysis of LeDET2 expressed in COS-7 cells. 5α-Reductase activity for progesterone, testosterone, androstenedione and campestenone of LeDET2 expressed in COS-7 cells. Substrates at the concentration of 50 nM were incubated with the lysate of transfected and non-transfected cells. Specific 5α-reductase activity was calculated subtracting the endogenous 5α-reductase activity of non-transfected cells.

Table 2
Kinetic constants for progesterone, testosterone, androstenedione of LeDET2 protein expressed in COS-7 cells

Substrate	K_m (μM)	V_{max} (nmol min^{-1} mg protein^{-1})
Progesterone	0.75 ± 0.05	0.34 ± 0.01
Androstenedione	26.34 ± 2.98	0.32 ± 0.01
Testosterone	44.60 ± 0.87	0.21 ± 0.01

Each K_m value represents the average of three different experiments carried out in different days. All values are reported as mean ± S.D.

COS-7 cells for progesterone were determined using increasing concentrations of substrate (0.01–20 μM) and 1 mM NADPH as cofactor. The apparent K_m value calculated was 0.4 μM and the V_{max} was 1.8 pmol min^{-1} mg $protein^{-1}$. The apparent K_m value determined for AtDET2 expressed in COS-7 cells is in agreement with that previously reported for AtDET2 expressed in human embryonic kidney 293 cells [6].

To further characterize the activity of recombinant LeDET2, some inhibitors of human 5αRs were used. It has been demonstrated that AtDET2 was inhibited by the 4-azasteroid 4-MA [6] and that several inhibitors of human 5αRs were active towards 5α-reductases in *S. malacoxylon* [8]. To determine if the enzyme LeDET2 was inhibited by these compounds, cell lysates containing recombinant LeDET2 were incubated with 1 μM progesterone as substrate and some steroid (4-MA, VG106, PD91, PD17, Finasteride) and non-steroid (AS601811, AFA27, AFA76, AFA131, AFA192) inhibitors at the concentration of 10 μM (Fig. 5). Results are shown in Fig. 6. All the inhibitors were active towards the recombinant LeDET2 enzyme. With the exception of finasteride, steroid inhibitors were more active than

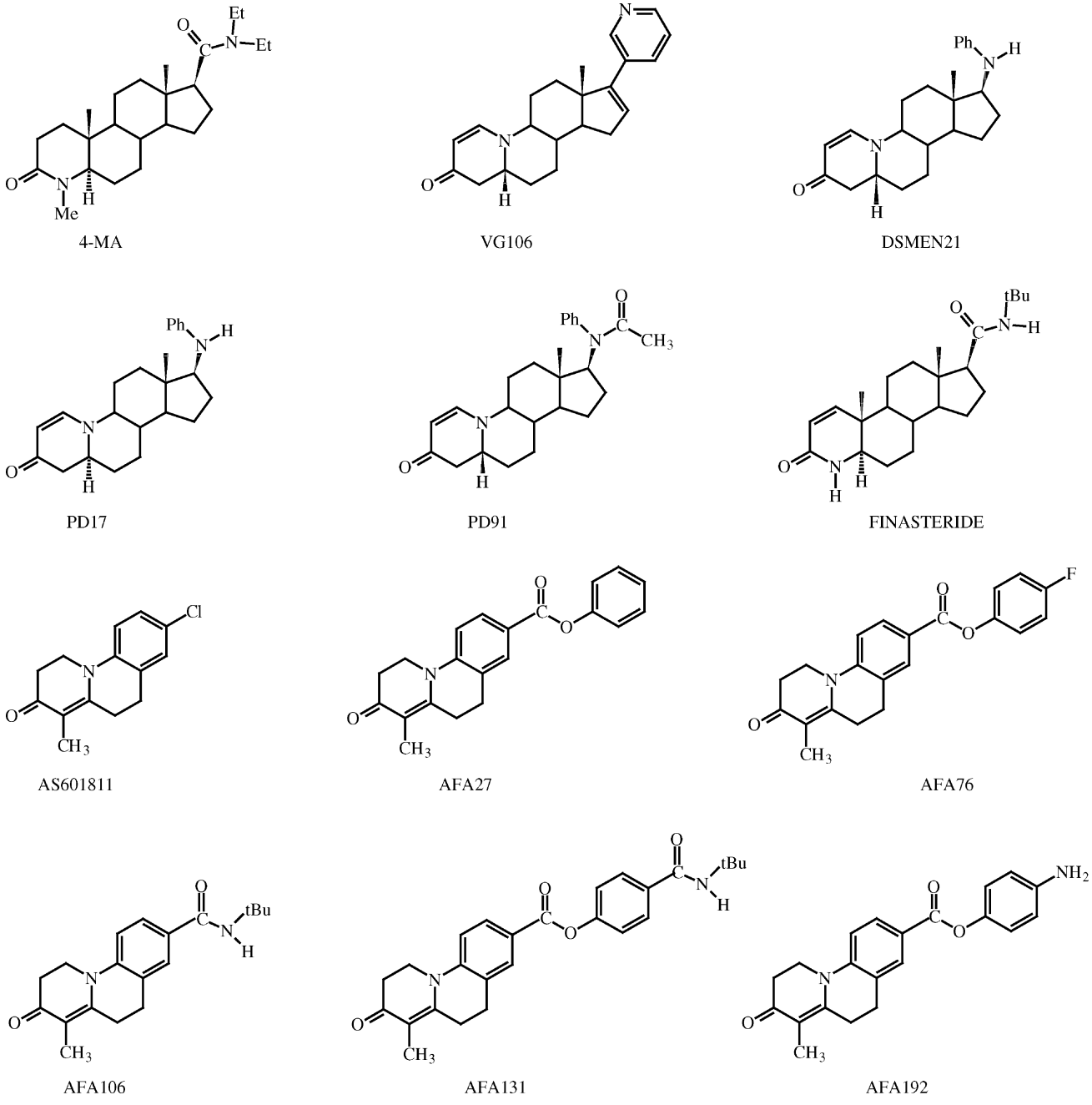

Fig. 5. Structures of steroid and non-steroid 5αRs inhibitors.

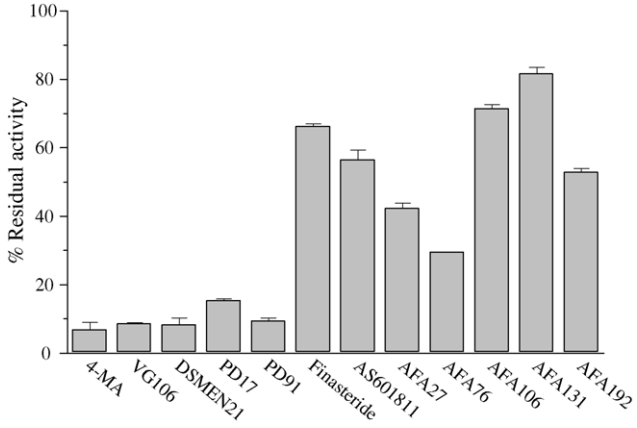

Fig. 6. Inhibition of LeDET2 expressed in COS-7 cells. Residual 5α-reductase activity (%) is shown at 10 μM concentration of each compound with 1 μM progesterone as substrate. Each column represents mean with S.D.

non-steroid molecules. AFA76 was the most potent inhibitor among non-steroid compounds tested and was used to characterize tomato 5α-reductase activity (in vitro and in vivo tests).

3.4. Effect of AFA76 on growth and morphology of tomato seedlings in the light and in the dark

In order to investigate the effect of inhibition of 5α-reductase in vivo, tomato seedlings were treated with the inhibitor AFA76. Preliminary experiments indicated that the highest inhibition effect was obtained at 20 μM concentration after 10 days of treatment. In addition, we observed a reduction of the growth of the seedlings exposed only to the vehicle (Et-OH) as expected. After 10 days of growth in the light or in the dark, the AFA76-treated and non-treated (only vehicle) seedlings were evaluated for morphological changes and the hypocotyl lengths were measured.

Fig. 7a shows the morphology of AFA76-treated and non-treated seedlings grown in the light and in the dark. Treated seedlings grown in the light exhibited a late development and in particular reduced hypocotyl and root elongation, reduced cotyledon expansion and the lack of the emergence of the first true leaves (seedlings treated longer than 10 days indicated a delay in the emergence of the first true leaves in comparison with the control). In the dark, AFA76 induced a phenotype with a short hypocotyl and closed cotyledons, similar to tomato BR-deficient mutants [2]. The reduction of hypocotyl length of AFA76-treated seedlings was more

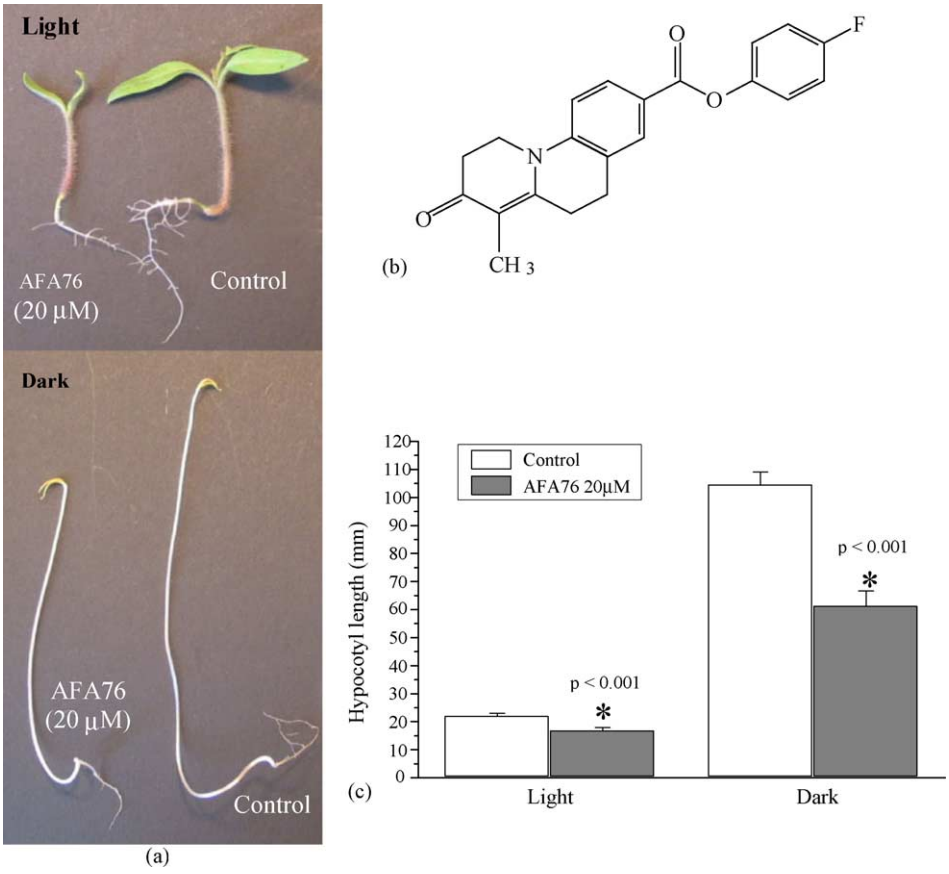

Fig. 7. Effect of AFA76 on tomato seedlings growth. (a) Morphology of untreated (Et-OH as vehicle) and AFA76-treated (20 μM) tomato seedlings (10-day-old) grown in the light (upper) and in the dark (down). (b) Structure of AFA76. (c) Effect of AFA76 on hypocotyl length of tomato seedlings grown in the light and in the dark. Data are the means ± S.E. obtained from 25 seedlings. Control: plants with Et-OH (vehicle).

evident in dark-grown seedlings (42%) than in light-grown seedlings (25%) as shown in Fig. 7c.

3.5. 5αR activity in tomato tissues

5α-Reductase activity was assayed in leaves, stems and roots of soil-grown tomato plants with campestenone, progesterone, testosterone and androstenedione as substrates. 5α-Reductase activity was measured with all substrates with the exception of androstenedione that in tomato tissues was metabolized to testosterone by a 17β-hydroxysteroid dehydrogenase (17β-HSD) activity. The formation of 5α-reduced steroids was linear over a 120 min period of incubation in leaves and over a 60 min period of incubation in stems and roots (data not shown).

Fig. 8a shows the results obtained measuring 5α-reductase activity with substrates at 10 nM concentration. The 5α-reductase activity was greatest in stems and relatively high in roots with campestenone reduced to 5α-campestanone at the highest levels. In leaves, 5α-reductase activity was lower than in other tissues and testosterone was the substrate reduced at the highest levels. Noteworthy, the ratio between the three products was not conserved in the analysed tissues suggesting the presence of more than one isoenzyme with 5α-reductase activity and different affinity for the substrates used. To test this hypothesis we investigated the biochemical behaviour of 5αR in tomato by determining the kinetic constants for campestenone in leaf homogenates. Fig. 8b shows the results obtained calculating the velocities of 5α-reductase activity with campestenone 0.01–10 μM. Velocities were plotted against *V/S* (Eadie–Hofstee transformation). The Eadie–Hofstee plot was nonlinear over the whole substrate concentration range, showing the presence of two enzyme activities. Two apparent K_m values were calculated from the Eadie–Hofstee plot: a K_m value of 0.05 μM for the enzyme with high affinity for campestenone and a K_m value of 4.2 μM for the enzyme with lower affinity for campestenone.

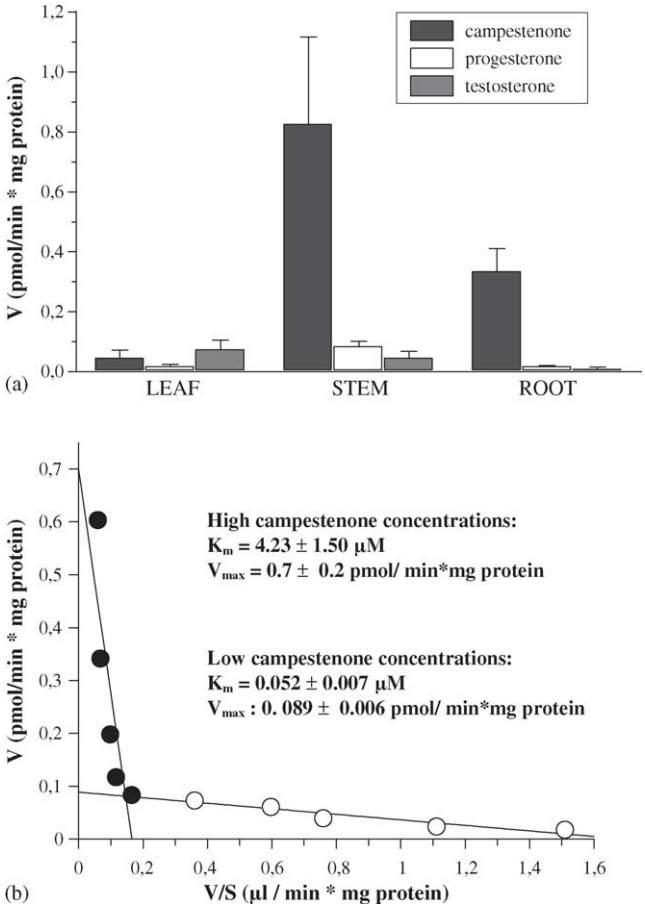

Fig. 8. 5α-Reductase activity in tomato tissues. (a) 5α-reductase activity in leaf, stem and root of tomato plants. 5α-Reductase activity was determined in the presence of 10 nM campestenone, progesterone and testosterone. Each column represents mean with S.D. (n = 2–4). (b) 5α-reductase activity in leaves of tomato plants. Velocities were obtained at 0.01–10 μM campestenone and 1 mM NADPH at 25 °C. The Eadie–Hofstee plot of *V* against *V/S* (campestenone concentration) is clearly non-linear in leaf homogenates and the kinetic constants of two 5α-reductase isoenzymes were calculated from these data (mean ± S.D.).

To further characterize 5αR activity in tomato tissues, the human 5αRs inhibitor AFA76 was used. 5αR activity in leaf, stem and root homogenates was measured in the presence of 10 μM AFA76 and 10 nM campestenone. The inhibitor was active in all tomato tissues but the residual 5αR activity was 50% in stems and roots while in leaves was 75%. The different activity of AFA76 further supports the hypothesis of the presence of two isoenzymes of 5αR in tomato.

3.6. 5αR activity in the Arabidopsis det2-1 mutant

To investigate whether more than one 5α-reductase enzyme was present also in *Arabidopsis*, we have assayed 5α-reductase activity in *Arabidopsis det2-1* mutant seedlings. In this mutant the substitution of lysine for Glu-204 totally inactivated the steroid 5α-reductase activity of AtDET2. We were able to measure appreciable levels of 5α-reductase activity in *det2-1* mutant seedling homogenates with campestenone and progesterone as substrates. We have also determined the apparent K_m and V_{max} values for campestenone and progesterone measuring 5α-reductase activity in the presence of 0.01–2 μM substrates (Fig. 9). The apparent K_m values calculated were 0.20 μM for campestenone and 0.06 μM for progesterone. These results demonstrate the presence of two isoenzyme of 5αR also in *Arabidopsis*. The "non-DET2" 5αR isoenzyme had higher affinity for progesterone than for campestenone.

4. Discussion

We have isolated and characterised *LeDET2*, the full-length cDNA encoding the homolog in *L. esculentum* of the *Arabidopsis* steroid 5α-reductase *DET2* (*AtDET2*).

The molecular cloning of *LeDET2* showed the existence of structural characteristics in the deduced amino acid sequence

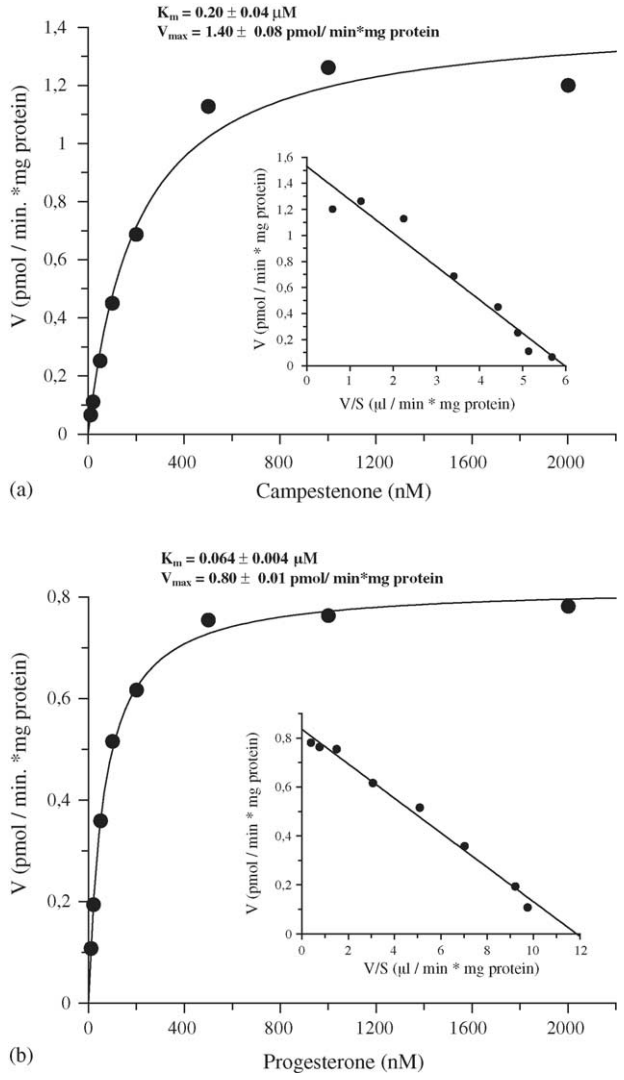

Fig. 9. 5α-Reductase activity in *Arabidopsis det2-1* mutant seedlings. Velocities were obtained at 0.01–2 μM campestenone (a) and progesterone (b), 1 mM NADPH, 25 °C. The figure shows the Michaelis–Menten plot and the Eadie–Hofstee transformation for each activity. The kinetic constants of 5α-reductase, reported as mean ± S.D., were calculated by non-linear regression analysis based on Michaelis–Menten equation. The Eadie–Hofstee plots were linear with both the substrates.

that are conserved among plant and animal steroid 5α-reductases. The amino acid residues absolutely required for 5α-reductase activity of mammalian enzymes that influence binding of co-factor and substrate, are well conserved in LeDET2. The LeDET2 amino acid sequence showed high homology to AtDET2. Although tomato diverged from *Arabidopsis* as much as 150 million years ago, early in the period of dicot diversification, genes encoding metabolism appear to evolve quite slowly [22].

To further characterise *LeDET2* gene, we investigated the expression in different tissues at different developmental stages. *LeDET2* was expressed, with little changes in transcript abundance, in all organs and tissues tested. In particular, the expression reached the highest level in the leaf of soil-grown plants. In contrast, the *LeDET2* transcripts were less abundant in stem, root and seed. To determine whether *LeDET2* encodes a functional 5α-reductase, *LeDET2* was transiently expressed in COS-7 cells. Recombinant LeDET2 was able to reduce substrates typical of mammalian steroid 5α-redutases (progesterone, androstenedione, testosterone) with the highest affinity for progesterone, but surprisingly campestenone, the natural substrate described for AtDET2, was reduced at very low levels. A similar biochemical behaviour was observed also for AtDET2 expressed in COS-7 cells. Recombinant AtDET2 reduced progesterone with an apparent K_m value of 0.4 μM, similar with that previously reported for AtDET2 expressed in a different mammalian cell line [6], but campestenone was not metabolized. The failure to detect enzyme activity with campestenone when LeDET2 or AtDET2 were expressed in a mammalian cell system may be due to some missing accessory molecule or to a different stereochemical conformation of the protein in this heterologous system. The same hypothesis was made when BRI1 transfected in 293T cells failed to dimerize in the presence of brassinolide [23]. Alternatively, campestenone could not be the favourite substrate for both LeDET2 and AtDET2. In fact, it has recently been demonstrated the existence, in *A. thaliana*, of a C-22 oxidation subpathway in brassinosteroids biosynthesis, implying that plant 5α-reductase can catalyze multiple 5α-reduction of 3-oxo-Δ^4 steroids including campestenone and 22-OH-campestenone [24]. Moreover, it has been described that C_{27} BRs can be synthesized from cholesterol via cholestenone, implying that cholestenone and 22-OH-cholestenone could also be substrates for plant 5α-reductase [24,25]. Therefore, plant 5α-reductase can catalyze 5α-reduction of many related sterols and the preferential metabolic pathway could be different from that at present described.

5α-Reductase activity of recombinant LeDET2 was also characterized using inhibitors of human 5αRs. The results of inhibition tests, performed with progesterone as substrate, indicated that all the inhibitors were active towards LeDET2. The steroid inhibitors, with the exception of finasteride, were more active than non-steroid molecules, in agreement with that previously reported for recombinant AtDET2 [6] and 5αRs in *S. malacoxylon* [8]. Similarly to AtDET2 [6] and *Solanum* 5α-reductases [8], LeDET2 was inhibited by several inhibitors of human 5αR system, pointing out the analogies between plant and mammalian 5αRs. AFA76, the most potent inhibitor among non-steroid compounds tested, was chosen for the inhibition tests on tomato seedlings because of its availability in amount convenient to perform the in vivo inhibition tests. In this study, we demonstrated that AFA76 induces morphological changes in tomato plants. The late emergence of the first true leaves observed in light-grown plants suggests a role of 5α-reductase in leaf development. In addition, the reduced hypocotyl and root elongation observed in AFA76-treated plants, further support the involvement of 5α-reductase in plant growth. Dark-grown AFA76-treated plants exhibited closed cotyledons and a reduced hypocotyl

length. These features are common to tomato BR biosynthesis mutants. These results indicate that the use of specific enzymatic inhibitors represents a valuable tool to investigate the function of enzymes in plant development.

The characterization of 5αR activity in tomato tissues was performed using leaves, stems and roots of soil-grown plants with the complete panel of steroidal substrates: campestenone, progesterone, testosterone and androstenedione. We were able to detect 5α-reductase activity with campestenone, progesterone and testosterone, while androstenedione was converted to testosterone implying a 17β-hydroxysteroid dehydrogenase (17β-HSD) activity. This is the first evidence of the presence of a 17β-HSD activity in plants. 17β-HSD is well known and characterized in mammals, in which a family of twelve enzymes essential in the biosynthesis of estrogens and androgens as well as in the modulation of hormone action have been described [26,27]. 17β-HSD was also found in bacteria [28], yeasts [29,30] and fungi [31,32]. The endogenous substrate/s and the role of plant 17β-HSD are exciting issues that could deserve further elucidation.

Differently from what we have observed for recombinant LeDET2 expressed in the heterologous system of COS-7 cells, campestenone was reduced to 5α-campestanone in tomato tissue homogenates. In particular, campestenone was 5α-reduced at the highest levels in stems and roots, the tissues with the greatest total 5α-reductase activity. In leaves, in which 5α-reductase activity was lower than other tissues, testosterone was the substrate 5α-reduced at the highest levels. These results of 5α-reductase activity are not correlated to those of *LeDET2* expression. In fact, the levels of *LeDET2* transcripts were higher in leaf than in stem and root. Although transcript levels, proteins, and enzymatic activity are not necessary correlated, the possibility exists that another isoenzyme contributes to the total 5αR activity. This hypothesis is supported by the observation that the ratio between the three products, 5α-campestanone, 5α-progesterone and 5α-dihydrotestosterone was not maintained in the tissues analysed suggesting the presence of more than one 5αR isoenzyme with different affinity for the substrates used. The determination of kinetic constants for campestenone in leaf homogenates confirms this hypothesis. The Eadie–Hofstee plot of the kinetic data was in fact nonlinear over the whole substrate concentration range, indicating the existence of two isoenzymes. The high-affinity isoenzyme had an apparent K_m 0.05 μM for campestenone and the low-affinity isoenzyme showed an apparent K_m 4.2 μM.

Inhibition tests with AFA76 were also performed in order to further characterize 5αR activity in tomato tissue homogenates. Residual 5αR activity in the presence of AFA76 and campestenone as substrate was 50% in stems and roots while in leaves was 75%. The different response to AFA76 in the tissues analysed corroborates the hypothesis of the existence of two 5αR isoenzymes in tomato.

We previously observed a similar biochemical behaviour, i.e. different kinetic characteristics and response to human 5αR inhibitors, for 5α-reductase activity in *S. malacoxylon* [8]. In this plant belonging to the *Solanaceae*, same tomato family, the presence of two isoenzymes was in fact biochemically demonstrated. Furthermore, we were able to demonstrate the presence of two 5αR isoenzymes also in *A. thaliana*. We have found 5α-reductase activity in *det2-1* mutant seedlings, in which AtDET2 enzyme was inactive. A residual concentration (10% versus wild type) of campestanol, a downstream product of 5α-campestanone in the brassinolide biosynthesis, was previously reported for the *Arabidopsis det-2* mutant. The authors hypothesised the existence of an alternative pathway or of a second 5α-reductase but they did not exactly identified the alternative step leading to the downstream product [33]. A similar hypothesis was recently made for *lk* mutant of pea [34]. However, the paper that precisely defines the blocked step in the *Arabidopsis det2* mutant did not report an alternative 5 alpha reductase activity [4]. We measured a 5α-reductase activity in *det2-1* seedling homogenates with both campestenone and progesterone as substrates, definitively demonstrating the presence of a second 5αR enzyme in *Arabidopsis*. The kinetic characterization of this 5αR activity showed that the "non-DET2" enzyme had higher affinity for progesterone (K_m = 0.06 μM) than for campestenone (K_m = 0.20 μM).

These results suggest that the existence of two 5αR isoenzymes is a general feature of the whole plant kingdom and highlights the similarities with the mammalian 5αR system in which the two 5αR isoenzymes have different affinity for the substrates and a differential response to inhibitors.

Acknowledgements

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