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Occurrence and conversion of progestogens and androgens are conserved in land plants

Glendis Shiko[1], Max-Jonas Paulmann[1], Felix Feistel[2], Maria Ntefidou[3], Vanessa Hermann-Ene[4], Walter Vetter[4], Benedikt Kost[3], Grit Kunert[2], Julie A. Z. Zedler[5], Michael Reichelt[2], Ralf Oelmüller[1] and Jan Klein[1]

[1]Department of Plant Physiology, Matthias-Schleiden-Institute for Genetics, Bioinformatics and Molecular Botany, Friedrich Schiller University Jena, Jena, 07743, Germany; [2]Department for Biochemistry, Max Planck Institute for Chemical Ecology, 07743, Jena, Germany; [3]Cell Biology, Department of Biology, University Erlangen-Nuremberg, 91058, Erlangen, Germany; [4]Institute of Food Chemistry, University of Hohenheim, 70599, Stuttgart, Germany; [5]Synthetic Biology of Photosynthetic Organisms, Matthias Schleiden Institute for Genetics, Bioinformatics and Molecular Botany, Friedrich Schiller University Jena, 07743, Jena, Germany

Author for correspondence:
Jan Klein
Email: jan.klein@uni-jena.de

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Summary

- Progestogens and androgens have been found in many plants, but little is known about their biosynthesis and the evolution of steroidogenesis in these organisms. Here, we show that the occurrence and biosynthesis of progestogens and androgens are conserved across the *viridiplantae* lineage.
- An UHPLC-ESI-MS/MS method allowed high-throughput analysis of the occurrence and chemical conversion of progestogens and androgens in 41 species across the green plant lineage.
- Dehydroepiandrosterone, testosterone, and 5α-dihydrotestosterone are plants' most abundant mammalian-like steroids. Progestogens are converted into 17α-hydroxyprogesterone and 5α-pregnane-3,20-dione. Androgens are converted into testosterone and 5α-dihydrotestosterone. 17,20-Lyases, essential for converting progestogens to androgens, seem to be most effective in monocot species.
- Our data suggest that the occurrence of progestogens and androgens is highly conserved in plants, and their biosynthesis might favor a route using the Δ^4 pathway.

Introduction

Sterols and steroids, which are produced in most organisms, are important for controlling membrane fluidity. Chemically, steroids are tetracyclic triterpenoids with a sterane skeleton (Fig. 1a, e.g. dehydroepiandrosterone, DHEA, in Fig. 1b).

A class of steroids in plants brassinosteroids act as phytohormones and are involved in developmental, biochemical processes and (a) biotic stress responses (Peres *et al.*, 2019). Progesterone (pregn-4-ene-3,20-dione; PO), which influences mammalian pregnancy, was found in *viridiplantae*, but its physiological role in plants is not fully understood. PO, first reported in plants in the 1960s (Bennett & Heftmann, 1965; Gawienowski & Gibbs, 1968), influences hypocotyl elongation and stress responses (Iino *et al.*, 2007; Genisel *et al.*, 2013; Shpakovski *et al.*, 2017; Hao *et al.*, 2019) and PO-binding proteins have been reported (Yang *et al.*, 2005). Progestogens, especially PO, are a precursor for 5β-cardenolides (Klein *et al.*, 2022). Despite advancements in characterizing plant progestogens and androgens (Janeczko, 2021), their evolutionary origin, biosynthesis, and physiological functions are largely unknown. To describe progestogen and androgen biosynthesis, steroidogenesis in plants will be used according to Lindemann (2015), although other steroids are also present in plants.

Mammalian steroidogenesis is initiated by **CYP11A1** (Fig. 2) in mitochondria (Miller & Auchus, 2011). This sterol monooxygenase catalyzes the three sequential hydroxylations of cholesterol (CO) to pregnenolone (PR, Simpson & Boyd, 1967). A hydroxylation at C20 follows a hydroxylation at C22. The third hydroxylation cleaves the side chain between C20 and C22, resulting in the C_{21}-steroid PR (Miller & Auchus, 2011). Plants contain only small amounts of C_{27}-(e.g. CO) compared with C_{29}-sterols (e.g. β-sitosterol), and both molecules can be converted into PR (Bennett & Heftmann, 1966; Bennett *et al.*, 1969). In plants, sterol side chain cleavage (SCC) activity was found in enriched mitochondrial protein fractions of *Digitalis purpurea* L. seedlings (Pilgrim, 1972). Phenobarbital treatment boosted SCC activity, indicating the P450-nature (CYP) of the SCC enzyme(s) (SCCE; Palazón *et al.*, 1995). It was shown recently, that CYP87A3 is an SCCE of *Digitalis lanata* EHRH. (*D. lanata*; Carroll *et al.*, 2022).

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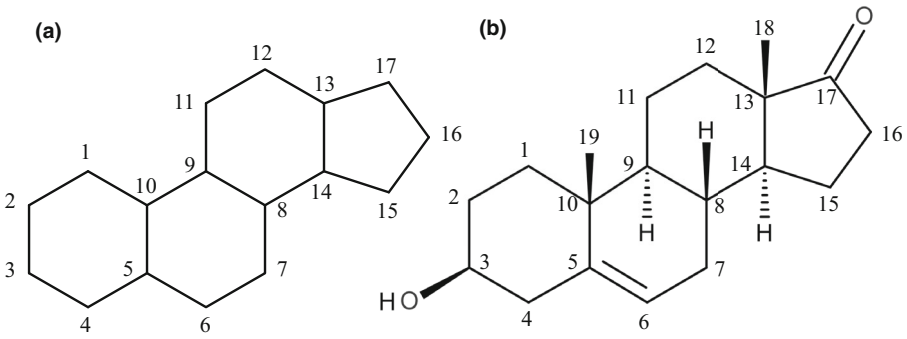

Fig. 1 Structure and numbering of carbon atoms in the sterane skeleton of steroids. The figure depicts the sterane skeleton of steroids with numbered carbon atoms (a). In dehydroepiandrosterone (DHEA), the sterane skeleton is additionally substituted with a methyl group at C10 and C13, a hydroxyl group at C3, and an oxo moiety on C17, along with a Δ^5 double bond (b).

The second mammalian steroidogenesis enzyme is the steroid 17α-hydroxyhydroxylase **CYP17A1** (Fig. 2). It catalyzes the 17α-hydroxylation of PR and PO to give 17α-hydroxypregnenolone (17α-OHPR) and 17α-progesterone (17α-OHPO), respectively (Fig. 2). Moreover, CYP17A1, showing 17,20-lyase activity, catalyzes the conversion of 17α-OHPR and 17α-OHPO into the androgens DHEA and androstenedione (AD; Porubek, 2013).

Mammalian DHEA conversion into AD is mainly catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD) 3B (**HSD3B**). 3β-HSD enzymes are found in *Arabidopsis thaliana* (L.) HEYNH. (*A. thaliana*, e.g. At2g47140 (termed SDR5; Witt, 2008)), as well as in the cardenolide-containing plant *D. lanata* (Herl *et al.*, 2007) and *Erysimum crepidifolium* RCHB. (*E. crepidifolium*; Munkert *et al.*, 2014). Based on a TargetP analysis of *D. lanata* SDR5 and 3β-HSD, these enzymes are assumed to be located in the cytosol. PR, 17α-OHPR, and DHEA are substrates of the *A. thaliana* enzymes (Witt, 2008), but the effects of these enzymes on steroid levels and composition *in planta* are unknown. 3β-HSD activity differs in mammalian and plant steroidogenesis: isoprogesterone is the main product of plant 3β-HSDs (Finsterbusch *et al.*, 1999), while human HSD3B has also Δ5-3-ketosteroid isomerase (KSI) activity. An unidentified *D. lanata* KSI was purified and characterized (Meitinger *et al.*, 2015, 2016).

In humans, the reduction of AD to testosterone (TO) is catalyzed by **HSD17B3,** a 17β-hydroxysteroid dehydrogenase (17β-HSDs; Miller & Auchus, 2011). 3β-HSD1 and 3β-HSD2 of *E. crepidifolium* (a cardenolide-containing Brassicaceae) showed both 3β-HSD activity and 17β-activity (Munkert *et al.*, 2014). Finally, TO is converted into 5α-dihydrotestosterone (DHT; Andersson & Russell, 1990) by a steroid-5α-reductase (**SRD5A**), whereas in plants, DET2 (At2g38050), an enzyme in brassinosteroid biosynthesis (Fujioka *et al.*, 1997), carries out this conversion. While these enzymes can functionally replace each other (Li *et al.*, 1997), it is unclear whether DET2 is involved in the transformation of TO into DHT and PO into 5α-pregnane-3,20-dione in plants (5α-dihydroprogesterone; DHP).

Plant steroidogenesis is not well characterized, and most records are from 5β-cardenolide-containing plants such as *D. lanata* or *E. crepidifolium*. Here, we systematically analyzed steroidogenesis in 41 plant species. We show that progestogen and androgen biosynthesis is present and conserved across plant lineages and postulate a steroidogenesis pathway for *A. thaliana*.

Materials and Methods

Plant material used and growth conditions

Arabidopsis thaliana Col-0 seeds were surface sterilized for 8 min (sterilization solution: 5% chlorine bleach, and 20% *N*-lauroylsarcosine sodium salt, Merck KGaA, Darmstadt, Germany) and sowed on MS medium (Murashige & Skoog, 1962; Supporting Information Table S1). After stratification (2 d), plants were cultivated in long-day (16 h : 8 h, light : dark) conditions with light intensity of 60–70 μM m^{-2} s^{-1} (22°C). Twelve-day-old seedlings of *A. thaliana* were harvested. *Chlamydomonas reinhardtii* P. A. DANGEARD (*C. reinhardtii*) strain CC-4533 (cw15, mt-) was grown in TAP medium (Kropat *et al.*, 2011) using shake flasks (180 rpm, room temperature, natural light) for 10 d. *Hordeum vulgare* L. cv 'Golden Promise' (*H. vulgare*) was germinated and cultivated (same conditions as *A. thaliana*) on watered cotton and harvested after 10 d. Somatic embryos of *Larix decidua* MILL. (*L. decidua*, clone 4/93) were cultured on MSG1 medium (Kraft & Kadolsky, 2018) at 22°C and in dark. Cells were harvested after 4 wk of continuous culture. Protonema of *Physcomitrium patens* (HEDW.) BRUCH & SCHIMP. (*P. patens*) ecotype Grandsen (available in our laboratory since 2012; Ashton & Cove, 1977) were maintained on BCDA medium (Cove *et al.*, 2009), as described previously by Le Bail *et al.* (2019) and harvested after 10 d. Gametophores of *P. patens* were induced on BCD medium (BCDA medium without $[NH_4]^+$ tartrate) and harvested after 10 d. *Plantago lanceolata* L. (*P. lanceolata*) seeds were collected in Obereuerheim (Grettstadt, Bavaria, Germany). Plants of *P. lanceolata*, *Hyacinthus orientalis* L. cv 'Pink Pearl' (*H. orientalis*), *Olea europaea* L. (*O. europaea*), *Allium schoenoprasum* L. (*A. schoenoprasum*), *Petroselinum crispum* (MILL.) FUSS (*P. crispum*), and *Convallaria majalis* L. (*C. majalis*) were propagated in the glasshouse in (Plant Physiology, Jena, Germany). Leaves were harvested from 8-wk-old plants. A clone of *Spirodela polyrhiza* (L.) SCHLEID. (*S. polyrhiza*, clone 9509) was prepared from the Friedrich Schiller University Jena stocks and cultivated as described by Appenroth *et al.* (2018; Table S1). Leaf material of *Aspidistra elatior* KER GAWL. (*A. elatior*), *Azolla filiculoides* LAM. (*A. filiculoides*), *Cycas revoluta* THUNB. (*C. revoluta*), *Digitalis grandiflora* MILL. (*D. grandiflora*), *Dioscorea bulbifera* L. (*D. bulbifera*), *Drimia maritima* (L.) STEARN

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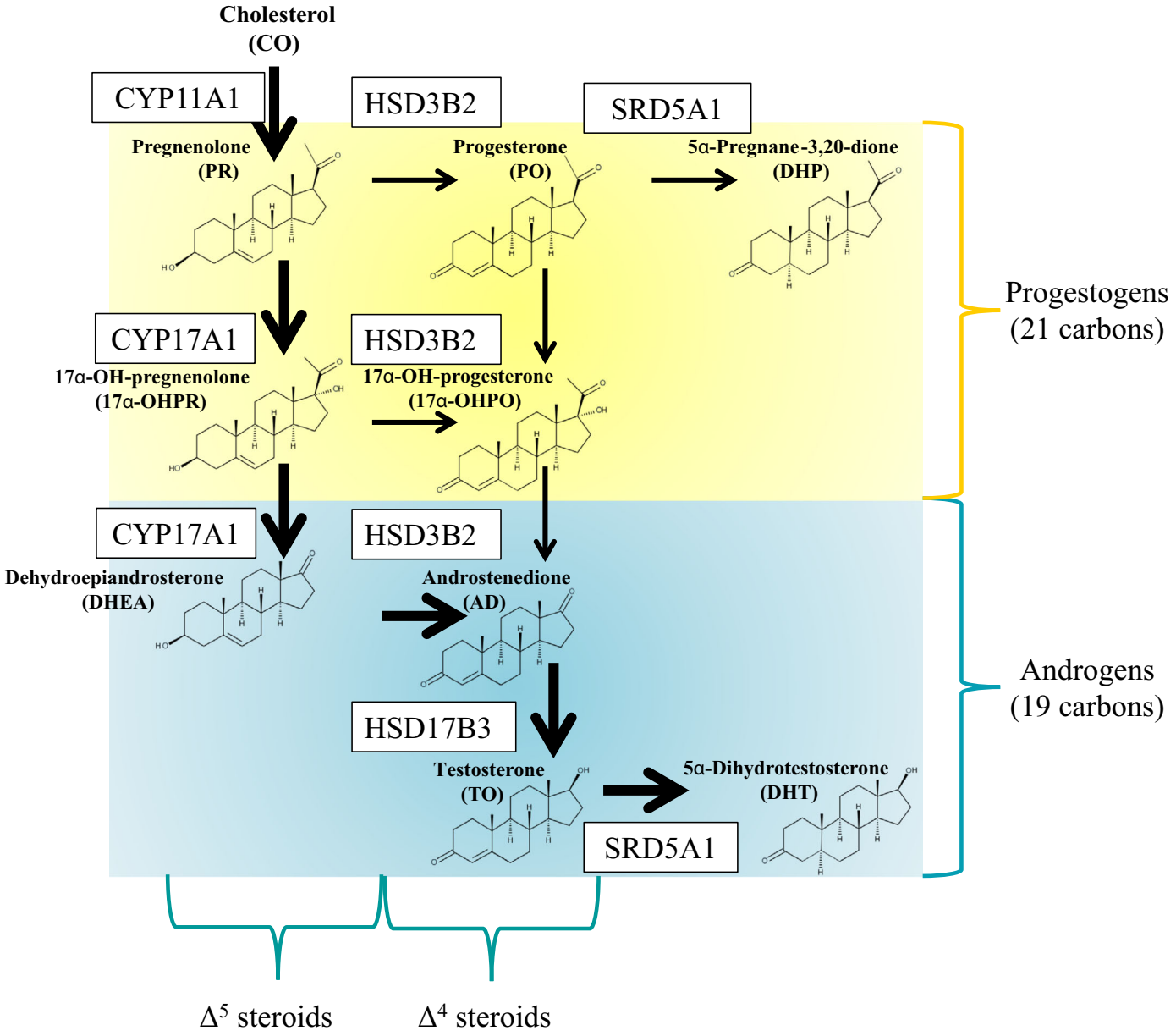

Fig. 2 Human steroidogenesis. Conversion of cholesterol (CO) into progestogens (yellow) and androgens (turquoise). CYP11A1 catalyzes three sequential hydroxylations of CO, which leads to the formation of pregnenolone (PR) and isocapronal (not shown). PR, the first progestogen in mammalian steroidogenesis, is converted into progesterone (PO) by HSD3B2, a 3β-hydroxysteroid dehydrogenase (3β-HSD). CYP17A1 converts PR and PO into 17α-hydroxypregnenolone (17α-OHPR) and 17α-hydroxyprogesterone (17α-OHPO) and also acts as a 17,20-lyase that converts 17α-OHPR into dehydroepiandrosterone (DHEA) and 17α-OHPO into androstenedione (AD). Thus, CYP17A1 synthesizes the first androgens in human steroidogenesis. HSD17B3, a 17β-hydroxysteroid dehydrogenase (17β-HSD), turns AD into the androgen testosterone (TO). PO and TO can be reduced by SRD5A1, a steroid 5α-reductase (5α-StR), into 5α-pregnane-3,20-dione (DHP) or 5α-dihydrotestosterone (DHT). Overall, five enzymes convert CO into all known progestogens and androgens. The main steroidogenesis pathway in mammals is depicted with bold arrows.

(*D. maritima*), *Freycinetia cumingiana* GAUDICH. (*F. cumingiana*), *Gnetum gnemon* L. (*G. gnemon*), *Nymphaea* hybrid 'Director Moore' (*N. hybrida*), *Pinus pinea* L. (*P. pinea*), *Ruscus aculeatus* L. (*R. aculeatus*), *Tacca chantrieri* ANDRÉ (*T. chantrieri*), *Taxaracum officinalis* L. (*T. officinalis*), *Taxus baccata* L. (*T. baccata*), and *Tulipa gesneriana* L. (*T. gesneriana*) were harvested in April 2021 in the Botanical Garden of Jena (Germany). Harvested plants were frozen in liquid nitrogen and used for steroid detection. Dried material of *Althaea officinalis* L. (*A. officinalis*), *Betula pendula* ROTH (*B. pendula*), *Chlorella vulgaris* BEIJ. (*C. vulgaris*), *Equisetum arvense* L. (*E. arvense*), *Eucalyptus globulus* LABILL. (*E. globulus*), *Fagopyrum esculentum* MOENCH (*F. esculentum*), *Galium odoratum* (L.) SCOP. (*G. odoratum*), *Gingko biloba* L. (*G. biloba*), *Laurus nobilis* L. (*L. nobilis*), *Lycopodium clavatum* L. (*L. clavatum*), *Melilotus officinalis* (L.) PALL. (*M. officinalis*), *Rubus fruticosus* agg. L. (R.

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fruticosus), *Salix alba* L. (*S. alba*), and *Vaccinium myrtillus* L. (*V. myrtillus*) was ordered from Wild Herbs s.r.o. (Rimov, Czech Republic).

Steroid treatment

Pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, and testosterone (all steroids from Merck KGaA) were added to culture media to a final concentration of 0.03 mM from 25 mM stocks in dimethyl sulfoxide (DMSO). Plant material was harvested after 2, 4, 6, and 8 d. DMSO was used as a mock treatment.

Transformation of *Arabidopsis thaliana*

The nucleotide sequence of the human CYP11A1 protein (Accession: NP_000772.2) was codon-optimized using the codon usage table of *H. vulgare* (https://www.genscript.com/codon-opt.html; Table S2). The plant-optimized human *CYP11A1* (*poCYP11A11*) gene was custom-synthesized and inserted into the vector pCAMBIA1301 by Biocat (Germany; Fig. S1). *A. thaliana* Col-0 was transformed using *Agrobacterium tumefaciens* (*A. tumefaciens* strain GV3101)-mediated floral-dip transformation (Clough & Bent, 1998) as described by Klein *et al.* (2021). Seeds were germinated on MS medium containing 25 mg l^{-1} Hygromycin B (Carl Roth GmbH, Karlsruhe, Germany) and 250 mg l^{-1} Cefotaxim (Duchefa Biochemie, Haarlem, the Netherlands). Transformants were screened by PCR using the primers hptII (Table S3).

Transformation of *Physcomitrium patens*

poCYP11A1 was amplified using the primers pFAU580_CYP11A1 (Table S3) and purified. pFAU580 was purified from DH5α cells using GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA). The plasmid and the amplified *poCYP11A1* sequence were digested with NotI-HF and EcoRI-HF (New England Biolabs, Ipswich, MA, USA) and ligated using T4 ligase (New England Biolabs). The NucleoSnap Plasmid Midi Kit (Macherey-Nagel, Düren, Germany) was used for large-scale plasmid preparation. *P. patens* was transformed by polyethylene glycol (PEG)-mediated transformation as described previously (Le Bail *et al.*, 2019) and based on Cove *et al.* (2009). Single lines were genotyped by PCR of genomic DNA. The *RIC* gene of *P. patens* was amplified to confirm successful DNA preparation (primers FAU-C123 and FAU-B757), while the primers for *poCYP11A1* gene amplification were used to detect transgenic moss lines.

DNA isolation and PCR

DNA of *A. thaliana* and *P. patens* was isolated using CTAB (Carl Roth GmbH, Germany) based method as used in Klein *et al.* (2022). Twenty µl reactions contained 2 µl genomic DNA, 2 µl of 10 µM of each primer (100 µM), 2 µl dNTPs (10 mM; Thermo Fisher Scientific), 0.2 µl DreamTaq DNA Polymerase and 2 µl DreamTaq DNA Polymerase buffer (Thermo Fisher Scientific). PCR program was set as follows: preincubation at 95°C for 180 s, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, 68°C for 45 s, and final amplification at 68°C for 150 s.

RNA isolation and qPCR

RNA of *A. thaliana* and *P. patens* was isolated using TRIzol® Reagent (Invitrogen™ by Thermo Fisher Scientific), as described previously (Chomczynski, 1993). RNA was transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) was performed in a Bio-Rad CFX96 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using 96-well plates. Twenty µl reactions with 1 µl cDNA, 2 µl dNTPs (10 mM; Thermo Fisher Scientific), 1 µl EvaGreen® Plus Dye (Biotium, Fremont, CA, USA), 2 µl of each primer (100 µM), 0.2 µl DreamTaq DNA Polymerase, and 3.4 µl DreamTaq DNA Polymerase buffer (Thermo Fisher Scientific) were used. qPCRs were performed with a preincubation at 95°C for 180 s, followed by 40 cycles at 95°C for 10 s, 60°C for 50 s, and 72°C for 60 s. Primers for qPCR can be found in Table S3. Three biological replicated were used for each qPCR.

Steroid quantification

Flash-frozen plant material was lyophilized (Alpha 1–4 LD plus; Martin Christ Gefriertrocknungsanlagen, Osterode im Harz, Germany) at 0.22 mbar and −35°C for 24 h. Dried material was pulverized, and 20 mg aliquots were extracted with 1 ml of extraction buffer (80% MeOH in ddH_2O; 100 ng ml^{-1} progesterone-D_9, Merck KGaA, as internal standard). Samples were vortexed for 60 s and sonicated (Sonorex Super RK 100 H; BANDELIN electronic, Berlin, Germany) for 5 min at 22°C. Crude extracts were centrifuged (HERMLE ZK233-M2; HERMLE Labortechnik GmbH, Gosheim, Germany) for 60 s at 16 000 ***g***, and supernatants used for the following analysis.

A volume of 2 µl was injected into an Agilent 1260 infinity II LC system, consisting of a binary pump G7112B, an autosampler G7167A, and a column thermostat G7116A (Agilent Technologies, Santa Clara, CA, USA) without preconcentration or filtering. Chromatographic separation was conducted on a ZORBAX Eclipse XDB-C_{18} column (50 × 4.6 mm, 1.8 µm) from Agilent Technologies. A binary solvent system was used as mobile phase consisting of (A) 0.05% formic acid in water and (B) acetonitrile with a constant flow rate of 1.1 ml min^{-1} at 20°C column temperature. The following gradient was applied: 0–0.5 min, 60% A; 0.5–5 min, 60–10% A; 5–5.05 min, 10–0% A; 5.05–6.5 min, 0% A; 6.5–6.55 min, 0–60% A; 6.55–9 min, 60% A. The column outlet was connected to a QTRAP 6500+ triple quadrupole mass spectrometer (AB Sciex LLC, Framingham, MA, USA). The Turbo Spray IonDrive ion source was running in positive ionization mode with 5500 V ion spray voltage and 650°C turbo gas temperature. The curtain gas was set to 40 psi, the collision gas to 'medium', and both ion source gases 1 and 2 were set to 70 psi. Scheduled multiple reaction monitoring (scheduled

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Table 1 Establishment and validation of an UHPLC–MS-based method for detection and quantification of progestogens and androgens in plants using an LC–MS/MS (HPLC 1260 (Agilent Technologies)-QTRAP6500 (SCIEX)) in positive ionization mode.

Steroid	Usage	*m/z*	Rt in min	CE in V	LD in ng ml^{-1}	LQ in ng ml^{-1}	Recovery in %	Precision in %	*f*
PR	Quantifier	299 → 281	4.29	15	2.5	80	74.7 ± 6.8	92.7 ± 2.5	0.49
	Qualifier	317 → 299		13			69.9 ± 15.3	92.3 ± 1.6	
PO	Quantifier	315 → 97	4.43	25	0.5	0.5	91.9 ± 14.3	94.0 ± 2.2	1.23
	Qualifier	315 → 109		30			91.7 ± 14.9	93.9 ± 2.3	
DHP	Quantifier	317 → 299	5.31	17	2	160	72.5 ± 19.7	87.7 ± 1.0	0.23
	Qualifier	317 → 281		19			72.0 ± 18.5	88.7 ± 1.5	
17α-OHPR	Quantifier	315 → 297	2.80	13	5	10	95.7 ± 4.1	94.6 ± 2.1	0.11
	Qualifier	333 → 297		13			92.5 ± 7.2	93.1 ± 4.2	
DHEA	Quantifier	289 → 271	3.01	9	10	10	92.3 ± 17.0	94.8 ± 2.2	0.08
	Qualifier	289 → 253		15			97.4 ± 16.7	94.3 ± 2.3	
17α-OHPO	Quantifier	331 → 97	3.15	27	0.5	0.5	96.1 ± 6.5	92.1 ± 1.7	0.57
	Qualifier	331 → 109		30			94.4 ± 6.4	92.5 ± 1.6	
AD	Quantifier	287 → 211	3.07	27	5	5	96.8 ± 4.0	94.2 ± 1.6	0.09
	Qualifier	287 → 173		29			98.6 ± 6.9	94.1 ± 1.7	
TO	Quantifier	289 → 97	2.58	25	0.5	0.5	97.4 ± 4.8	98.1 ± 0.7	1.34
	Qualifier	289 → 109		30			96.9 ± 4.4	98.3 ± 0.5	
DHT	Quantifier	291 → 255	3.45	21	0.5	64	83.7 ± 2.2	92.6 ± 2.8	0.84
	Qualifier	291 → 273		19			88.3 ± 9.1	91.3 ± 5.0	
PO-D_9	Quantifier	324 → 100	4.43	29	0.5	0.5	86.9 ± 5.4	92.0 ± 1.3	–
	Qualifier	324 → 113		33			86.7 ± 4.9	92.2 ± 1.1	

Scheduled multiple reaction monitoring (scheduled MRM) was used to monitor analyte parent ion → product ion fragmentations. Retention time (Rt) and collision energy (CE) of analytes were determined. Limits of detection (LD), limits of quantification (LQ), precision, and response factors (*f*) were determined experimentally. Recovery was determined using 20 mg ml^{-1} lyophilized *A. thaliana* leaves as plant matrix to analyze the matrix effects of plant leaves. 17α-OHPO, 17α-hydroxyprogesterone; 17α-OHPR, 17α-hydroxypregnenolone; AD, androstenedione; DHEA, dehydroepiandrosterone; DHP, 5α-pregnane-3,20-dione; DHT, dihydrotestosterone; nona-deuterated progesterone, PO-D_9; PO, progesterone; PR, pregnenolone; TO, testosterone.

MRM) was used to monitor analyte parent ion → product ion fragmentations as described in Table 1. Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.6 software (Applied Biosystems, Waltham, MA, USA) was used for data acquisition and processing. Nona-deuterated progesterone (PO-D_9) was used as internal standard for quantification. Individual steroids' response factors (analyte × standard^{-1}) relative to the internal standard have been experimentally determined (Table 1).

A dilution series of steroids in 80% MeOH, including the internal standard, was prepared for method validation, starting with the previously determined detection limits for each steroid (Table S4). Measurements were conducted without plant matrices. Limit of detection (LD) and limit of quantification (LQ) were calculated. To minimize plant matrix effects (ME), we used this dilution series to extract plant material (three replicates) as described previously. Plant extracts were used to determine steroid recovery in plant matrices. To determine the precision, the standard deviation of three technical replicates of solution A (composition described in Table S4) was analyzed.

Cholesterol measurement

Lipophilic compounds were cold-extracted (three times 6 ml *n*-hexane, ultrasonic bath) from *c.* 0.2 g lyophilized material (mentioned previously), according to Sommer & Vetter (2020). Samples were concentrated by rotary evaporation and adjusted to a 4 ml volume. Saponification and trimethylsilylation were performed according to Schlag *et al.* (2022). For saponification, 2 ml aliquots were transferred into 6-ml test tubes, and the solvent was removed with nitrogen. After adding 1.8 ml of ethanol, 0.2 ml of aqueous potassium hydroxide (KOH, 50% w/w), and flushing tubes with nitrogen, samples were heated for 2 h at 80°C. The unsaponifiable matter was extracted from samples after cooling to room temperature with 2 ml *n*-hexane and adding 0.5 ml distilled water. The organic phase was washed two times with 1.8 ml of ethanol, 0.2 ml of aqueous potassium hydroxide (KOH, 50% w/w^{1}), and 0.5 ml of distilled water. The extract was washed with 1 ml of distilled water. One hundred µl aliquots were evaporated for trimethylsilylation, and 25 µl of distilled pyridine and 50 µl SILYL-991 solution were added. After heating samples for 30 min at 60°C, the solvent was removed with nitrogen, and the residue dissolved in 100 µl of 5α-cholestane solution (6 µg ml^{-1} in *n*-hexane). Trimethylsilylated cholesterol was quantified by gas chromatography coupled to mass spectrometry (GC/MS) operated in selected ion monitoring (SIM) mode via *m/z* 129 (base peak) and *m/z* 458 (molecular ion). A 6890 GC/5973 N MSD system (Hewlett-Packard/Agilent, Waldbronn, Germany) was used with an MPS 2 autosampler system (Gerstel, Mülheim, Germany) operated in splitless mode (1 µl; Schlag *et al.*, 2022). The Optima 5HT column (30 m, 0.25 mm i.d., 0.25 µm film thickness, Macherey-Nagel), initially held for 1 min at 55°C, was then ramped at 20°C min^{-1} to 240°C, at 12°C min^{-1} to 283°C, and finally at 15°C min^{-1} to 300°C and held for 11 min.

Statistical analysis

In order to test whether there was a correlation between cholesterol and progesterone on the one hand and cholesterol and

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pregnenolone on the other hand, the Spearman's rank correlation was applied.

In order to test whether certain analytes were accumulated in a plant after steroid treatments, several tests were applied. In cases where the analyte of interest could not be detected in plants of a certain treatment, present/absent data were used and Bernoulli generalized linear models were applied. Significance values were obtained by comparing the model with the explanatory variable with the model without the variable with a likelihood ratio test (Zuur *et al.*, 2009). In cases of more than two factor levels per treatment, factor level reduction was applied to test which treatments differ to the control plants (Crawley, 2013). In case plants of all treatments contained a certain analyte, actual concentrations were used, and the Welch two-sample *t*-test (for comparison of one treatment against a control), an ANOVA (for comparisons of more than two treatments), or the Kruskal–Wallis rank-sum test (for comparisons of more than two treatments, if normality of the residuals or variance homogeneity was not fulfilled) were applied. Which data and test were used for which combination of plant species and treatment can be found in Table S6.

The analyses were done using the R software v.4.2.3. (R Core Team, 2018).

Results

Establishing a highly sensitive and reproducible method to quantify progestogens and androgens in plants

We have developed a highly sensitive method based on ultra-high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) method for the relative quantification of progestogens and androgens from freeze-dried plant samples, based on Simerský *et al.* (2009). The adapted method requires only 20 mg of lyophilized material for sample preparation. It allows high-throughput analysis of plant samples for large-scale phylogenetic analysis and analytical support for molecular biology assays. We determined individual steroids' retention time and collision energy (Table 1). PO-D_9 was used as the internal standard, and response factors (f) of steroids relative to this internal standard were determined (Table 1). For method validation, we established LD, LQ, and precision (Table 1). Standard curves of quantifiers (Fig. S1) and qualifiers (Fig. S2) are depicted in the supporting information. Crude extracts of biological samples contain compounds that can cause ion suppression/enhancement during MS ionization (e.g. Matuszewski *et al.*, 2003; Remane *et al.*, 2010). Signal decreases due to matrix-induced ion suppression cause the imprecise calculation of naturally occurring low-abundance substances. Matrix effects (ME) were determined using *A. thaliana* leaves. Steroids were extracted from *A. thaliana* leaves in the presence of a dilution series of steroid standards (dissolved in 80% MeOH). The results were used to determine steroid recovery and allow for ME estimation. Using 20 mg lyophilized *A. thaliana* leaves extracted in 1 ml of solvent allowed a high recovery of all analyzed steroids. MEs did not negatively influence steroid quantification. The method should be compatible for a broad range of photosynthetic organisms; therefore, we compared internal standard (100 ng ml^{-1}) signal intensities in extracts of *A. thaliana* leaves, *A. thaliana* roots, *H. vulgare* leaves, *H. vulgare* roots, *S. polyrhiza* shoots, somatic embryos of *L. decidua*, *P. patens* protonema, and cells of *C. reinhardtii* (Table S5). The internal standard's signal intensities (as peak areas in counts) were comparable across all the tested plant matrices. Raw data of method validation are available (doi: 10.5281/zenodo.7696597).

Steroids are detectable at amounts between 0.5 and 10 ng ml^{-1} using the developed UHPLC-ESI-MS/MS method.

Occurrence of progestogens and androgens in plants

Progestogen and androgen profiles of 40 plant species were analyzed. Table 2 shows progestogens and androgens occurrence in green algae, mosses, club mosses, ferns, *Equisetidae*, and gymnosperms.

Androgens (DHEA) occurred already in green algae (*C. vulgaris* and *C. reinhardtii*), while land plants (starting with mosses) featured multiple progestogens and androgens. DHEA was found in all nonspermatophytes, and, apart from *E. arvense*, all analyzed terrestrial nonseed plants contained progestogens and androgens. Gymnosperms contained progestogens (*C. revoluta* and *P. pinea*) or androgens (*G. biloba* and *G. gnetum*). However, only *G. biloba* contained both androgens (DHEA and DHT) and 17α-OHPO (a progestogen and direct precursor of androgens in mammalian steroid biosynthesis). This raises the question of whether the biosynthesis of androgens occurs through progestogen conversion, as in mammals, or via independent production.

Two monocots (black bat flower *T. chantireri* and great duckweed *S. polyrhiza*) had neither progestogens nor androgens (Table 3). Based on a one-point calibration with a mixture of authentic standards, we show that monocots contain lower amounts of progestogens and androgens (Table S7) than other analyzed plants.

We show that dicots (Table 4) contained the broadest spectrum of progestogens (PR, PO, and 17α-OHPR) and androgens (DHEA, TO, and DHT). Especially PO (15 of 19 plants), DHEA (17 of 19 plants), and DHT (13 of 19 plants) are abundant in dicot leaf tissue.

We demonstrate that progestogens and androgens already occur in green algae, and their existence is conserved in plants, apart from some monocot species.

Steroid profiles of 5β-cardenolide-containing and 5β-cardenolide-free plants

5β-Cardenolide biosynthesis is thought to occur in leaves and depends on PO (Luckner & Wichtl, 2000). Differences in leaf steroid profiles between 5β-cardenolide-containing plants (e.g. *D. grandiflora*, a member of Plantaginaceae family, or *E. crepidifolium*, a member of Brassicaceae) and 5β-cardenolide-free plants of the same family (e.g. *P. lanceolata* for Plantaginaceae or *A. thaliana* for Brassicaceae) were unknown.

PO (*P. lanceolata*: 78.2 ± 4.6 ng g^{-1} dry weight (DW); *D. grandiflora*: 69.4 ± 1.4 ng g^{-1} DW) and especially PR (*P. lanceolata*: 1140 ± 89.4 ng g^{-1} DW; *D. grandiflora*: 208 ± 20.1 ng g^{-1}

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Table 2 Steroid profiles of green algae, mosses, club mosses, ferns, Equisetidae, and gymnosperms.

Species		PR	PO	17α-OHPR	DHEA	17α-OHPO	TO	AD	DHT
*C. vulgaris**	Green algae	–	–	–	✓	–	–	–	–
*C. reinhardtii**	Green algae	–	–	–	✓	–	–	–	–
*P. patens***	Mosses	–	✓	–	✓	–	✓	–	–
*L. clavatum****	Club mosses	–	✓	–	✓	–	✓	–	✓
*A. filiculoides****	Ferns	✓	✓	–	✓	–	–	–	–
*E. arvense****	Equisetidae	–	–	–	✓	–	–	–	✓
*C. revoluta*****	Gymnosperms Cycadales	✓	✓	–	–	–	–	–	–
*G. biloba*****	Gymnosperms Ginkgoales	–	–	–	✓	✓	–	–	✓
*G. gnetum*****	Gymnosperms Gnetales	–	–	–	✓	–	–	–	✓
*P. pinea*****	Gymnosperms Coniferales	✓	✓	–	–	–	–	–	–

The occurrence of steroids (17α-OHPO, 17α-hydroxyprogesterone; 17α-OHPR, 17α-hydroxypregnenolone; AD, androstenedione; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; PO, progesterone; PR, pregnenolone; TO, testosterone) was analyzed in green algae (*C. vulgaris* and *C. reinhardtii*), mosses (*P. patens*), club mosses (*L. clavatum*), ferns (*A. filiculoides*), Equisetidae (*E. arvense*) and gymnosperms (*C. revoluta*, *G. biloba*; *G. gnetum* and *P. pinea*). The following plant material was used: *, whole cell material; **, gametophyte; ***, shoot material; ****, leaf material. (✓), steroid was clearly detected; (–), indicates that the analyte was not detectable, that is, no or too low signal (S/N < 3) or absence of the respective qualifier signal.

Table 3 Steroid profiles of monocots.

Species		PR	PO	17α-OHPR	DHEA	17α-OHPO	AD	TO	DHT
H. vulgare	Poales	–	✓	–	✓	–	–	–	–
A. schoenoprasum	Asparagales	–	✓	–	✓	–	–	✓	–
C. majalis	Asparagales	✓	✓	✓	✓	–	✓		–
A. elatior	Asparagales	✓	✓	–	✓	–	–		–
R. aculeatus	Asparagales	✓	✓	–	–	–	–	✓	–
D. maritima	Asparagales	✓	✓	✓	–	–	–		–
H. orientalis	Asparageles	✓	✓	–	✓	–	–		–
T. gesneriana	Liliales	✓	✓	–	✓	–	–	–	–
F. cumingiana	Pandanales	–	✓	–	✓	–	–	✓	✓
D. bulbifera	Dioscoreales	–	–	–	✓	–	–	–	–
T. chantrieri	Dioscoreales	–	–	–	–	–	–	–	–
S. polyrhiza	Alismatales	–	–	–	–	–	–	–	–

We analyzed the occurrence of steroids (17α-OHPO, 17α-hydroxyprogesterone; 17α-OHPR, 17α-hydroxypregnenolone; AD, androstenedione; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; PO, progesterone; PR, pregnenolone; TO, testosterone) in leaf material of *H. vulgare*, *Allium schoenoprasum* (*A. schoenoprasum*), *Convallaria majalis* (*C. majalis*), *Aspidistra elatior* (*A. elatior*), *Ruscus aculeatus* (*R. aculeatus*), *Drimia maritima* (*D. maritima*), *Hyacinthus orientalis* (*H. orientalis*) *Tulipa gesneriana* (*T. gesneria*), *Freycinetia cumingiana* (*F. cumingiana*), *Dioscorea bulbifera* (*D. bulbifera*), *Tacca chantrieri* (*T. chantrieri*) and *Spirodela polyrhiza* (*S. polyrhiza*). (✓), steroid was clearly detected; (–), indicates that the analyte was not detectable, that is, no or too low signal (S/N < 3) or absence of the respective qualifier signal.

DW) levels were higher in 5β-cardenolide-free *P. lanceolata* than in the 5β-cardenolide-containing Plantaginaceae species. Both species contained comparable amounts of DHEA. Furthermore, *D. grandiflora* contained the androgen TO, and DHT was found in *P. lanceolata*.

In *A. thaliana* and *E. crepidifolium*, no PR was detected, and PO concentrations were much higher for *A. thaliana* (66.7 ± 35.1 ng g^{-1} DW) than for *E. crepidifolium* (4.2 ± 0.9 ng g^{-1} DW). In *E. crepidifolium*, the only androgen detected was TO, while *A. thaliana* leaves contained DHEA, TO, and DHT.

Cardenolide-containing monocot species are known. We compared steroids of *C. majalis* (lily-of-the-valley), a cardenolide-containing *Asparagaceae* (subfamily: Nolinoideae), with Nolinoideae species (*A. elatior* and *R. aculeatus*) steroids. While *R. aculeatus* contains much higher contents of PR (579 ng g^{-1} DW) and PO (496 ng g^{-1} DW) compared with *C. majalis* (PR: 145 ng g^{-1} DW; PO: 468 ng g^{-1} DW), this was not the case for the comparison of *C. majalis* and *A. elatior* (PR: 190 ng g^{-1} DW; PO: 448 ng g^{-1} DW). We analyzed and compared the steroid profile of the bufadienolide-containing *Asparagaceae* species *D. maritima* (subfamily: Scilloideae) and the cardenolide-free Scilloidea species *H. orientalis*. We observed slightly enhanced PR and PO concentrations in the cardenolide-free compared with the cardenolide-containing species (*D. maritima*: PR: 132 ng g^{-1} DW; PO 395 ng g^{-1} DW; *H. orientalis*: PR: 148 ng g^{-1} DW; PO: 462 ng g^{-1} DW).

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Table 4 Steroid profiles of dicots.

Species		PR	PO	17α-OHPR	DHEA	17α-OHPO	AD	TO	DHT
P. lanceolata	Lamiales	✓	✓	–	✓	–	–	–	✓
D. grandiflora	Lamiales	✓	✓	✓	✓	–	–	✓	–
O. europaea	Lamiales	–	–	–	✓	–	–	–	✓
A. thaliana	Brassicales	–	✓	–	✓	–	–	✓	✓
E. crepidifolium	Brassicales	–	✓	–	–	–	–	✓	–
M. officinalis	Fabales	–	✓	–	✓	–	–	–	✓
V. faba	Fabales	–	–	–	✓	–	–	–	–
G. odoratum	Gentianales	✓	✓	–	✓	–	–	–	✓
S. alba	Maphighiales	✓	✓	–	✓	–	–	✓	✓
B. pendula	Fagales	✓	✓	–	✓	–	–	–	✓
F. esculentum	Caryophyllales	✓	✓	–	✓	–	–	–	✓
E. globulus	Myrtales	–	–	–	✓	–	–	–	✓
V. myrtillus	Ericales	✓	✓	–	✓	–	–	–	✓
A. officinalis	Malvales	–	✓	–	✓	–	–	–	✓
R. fruticosus	Rosales	✓	✓	–	✓	–	–	–	✓
Taraxacum sect. *Ruderalia*	Asterales	–	✓	–	✓	–	–	–	–
P. crispum	Apiales	–	–	–	✓	–	✓	–	–
L. nobilis	Laurales	–	✓	–	✓	–	✓	–	✓
N. hybrida	Nymphaeales	–	✓	–	–	–	–	✓	–

The occurrence of steroids (17α-OHPO, 17α-hydroxyprogesterone; 17α-OHPR, 17α-hydroxypregnenolone; AD, androstenedione; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; PO, progesterone; PR, pregnenolone; TO, testosterone) was analyzed in leaf material of *Plantago lanceolata* (*P. lanceolata*), *Digitalis grandiflora* (*D. grandiflora*), *Olea europaea* (*O. europaea*), *Arabidopsis thaliana* (*A. thaliana*), *Erysimum crepidifolium* (*E. crepidifolium*), *Melilotus officinalis* (*M. officinalis*), *Galium odoratum* (*G. odoratum*), *Salix alba* (*S. alba*), *Betula pendula* (*B. pendula*), *Fagopyrum esculentum* (*F. esculentum*), *Eucalyptus globulus* (*E. globulus*), *Vaccinium myrtillus* (*V. myrtillus*), *Althaea officinalis* (*A. officinalis*), *Rubus fruticosus* agg. (*R. fruticosus*), *Taraxacum* sect. *Ruderalia*, *Laurus nobilis* (*L. nobilis*) and *Nymphaea* hybrid 'Director Moore' (*N. hybrida*). (✓), steroid was clearly detected; (–), indicates that the analyte was not detectable, that is, no or too low signal (S/N < 3) or absence of the respective qualifier signal.

Both cardenolide-containing monocots showed 17α-OHPR, which remained undetected in other species.

Cholesterol as putative sterol precursor for steroidogenesis

In plants, steroidogenesis, including sterol precursors, is barely characterized. In mammals, CO to PR conversion (Fig. 3a) initiates steroidogenesis. A recent study demonstrated the existence of CO in plants (Sonawane *et al.*, 2016). We analyzed the correlation between CO and PR or PO content in plants. There was no correlation between CO values (Table S7) and PR and PO values (Fig. 3b). Thus, it is not clear whether CO can act as a sterol precursor of plant steroidogenesis.

CO levels differed strongly between *A. thaliana* (CO content: 59 $\mu g\, g^{-1}$ leaf DW) and *P. patens* (CO content: 205 $\mu g\, g^{-1}$ protonemata DW). We expressed a plant-optimized version of the human CYP11*A1* (*poCYP11A1*) in both species using plant expression vectors pCAMBIA1301 for *A. thaliana* (Fig. S3a) and pFAU580 for *P. patens* (Fig. S3b). Plants were transformed using floral dip for *A. thaliana* and PEG-mediated transformation of *P. patens*. *A. thaliana* plants surviving hygromycin B treatment, and *P. patens* plants surviving G418 (Geneticin) treatment, were analyzed by qPCR. The primers against the poCYP11A1-sequence did not amplify unspecific products in wild-type, while the poCYP11A1-fragment was amplified transgenic plants (Fig. S4). Transgenic lines with high poCYP11A1 expression levels (exemplarily for *A. thaliana* in Fig. S4) were used for steroid measurements.

Consistent with three times higher CO levels in *P. patens* than in *A. thaliana* leaves, heterologous expression of *poCYP11A1* leads to PR and PO formation in the protonemata of all transgenic *P. patens* lines (Fig. S5) and DHP accumulation in two out of three tested lines. None of these effects were seen in *A. thaliana*. These data indicate that CO can act as a steroid precursor in plants.

Biotransformation of progestogens and androgens in plants

Progestogens and androgens were found in nearly all analyzed plant species, indicating steroidogenesis conservation during plant evolution. To test this, we treated green algae (*C. reinhardtii*), a gymnosperm species (*L. decidua*), a steroid-containing monocot species (*H. vulgare*), a steroid-free (no progestogens and no androgens) monocot species (*S. polyrhiza*), and a dicot species (*A. thaliana*) with PR, PO, 17α-OHPR, 17α-OHPO, DHEA, AD, and TO and analyzed steroid profiles (Fig. 4).

The results of this analysis are described following the four reaction steps from pregnenolone to dihydrotestosterone in human steroidogenesis, and the statistical analysis can be found in Table S6.

17α-hydroxylase and 17,20-lyase reactions

The first step in mammalian steroidogenesis is a 17α-hydroxylation of PR (Fig. 5a) or PO (Fig. 5b). This reaction is essential for mammalian androgen biosynthesis. Since some

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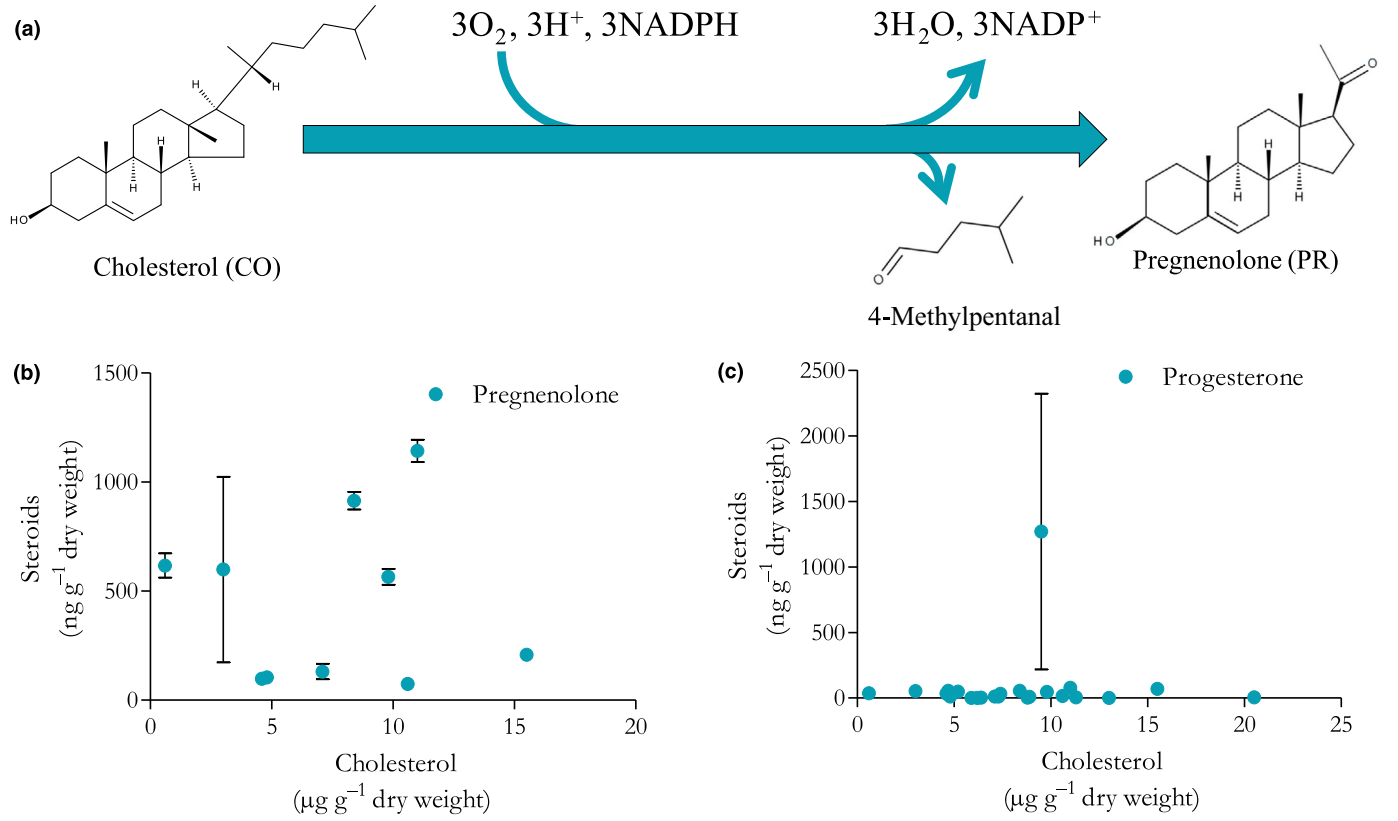

Fig. 3 Cholesterol as the precursor of plant steroidogenesis. (a) Cholesterol is converted into pregnenolone and 4-methylpentanal, a reaction that is catalyzed by CYP11A1 in mammals. (b) Therefore, we plotted cholesterol values against pregnenolone or (c) progesterone. Data points are averages of three replicates with error bars depicting SEM. No linear relationship between cholesterol and progestogen values and no Spearman correlation between cholesterol and pregnenolone (rho = 0.045, $P = 0.903$) was found. This was the same for cholesterol and progesterone (rho = −0.055, $P = 0.802$).

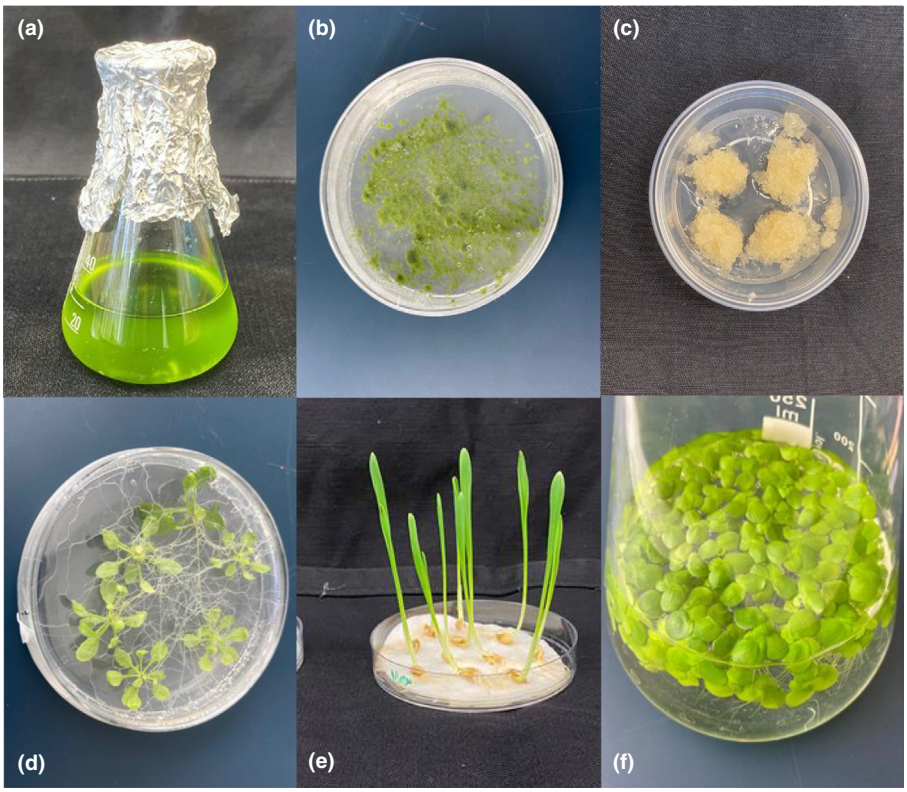

Fig. 4 Analysis of steroid conversion in the kingdom of plants. Cultures of *Chlamydomonas reinhardtii* (a; green algae), protonemata of *Physcomitrium patens* (b; moss), embryo cultures of *Larix decidua* (c; gymnosperm), *in vitro*-grown *Arabidopsis thaliana* plants (d; dicot), *Hordeum vulgare* seedlings (e; monocot), and aseptic *Spirodela polyrhiza* cultures (f; steroid-free monocot) were used. To analyze steroid conversion, medium or irrigation water (*H. vulgare*) was supplemented with steroids. Four days after treatment, the plant material was harvested, immediately flash-frozen, and lyophilized. Dried plant material was analyzed using nona-deuterated progesterone as an internal standard.

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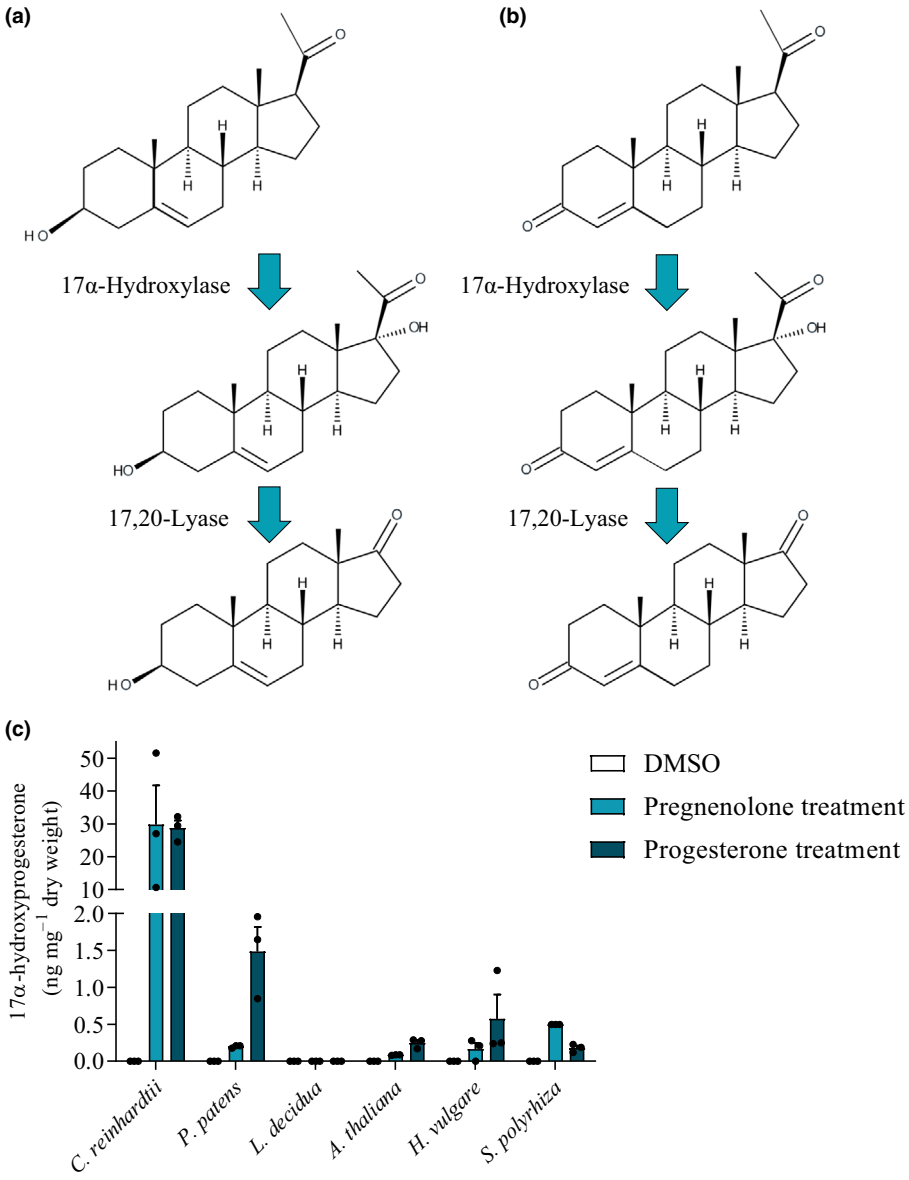

Fig. 5 17α-hydroxylase and 17,20-lyase reactions. The conversion of pregnenolone (a) and progesterone (b) by 17α-hydroxylases into 17α-hydroxypregnenolone, respectively, 17α-hydroxyprogesterone was analyzed. In mammals, both metabolites are accepted by 17,20-lyases and converted into dehydroepiandrosterone (a), respectively, androstenedione (b). In humans, these reactions will be performed by CYP17A1 while pregnenolone is the preferred substrate. *Chlamydomonas reinhardtii*, *Physcomitrium patens*, *Larix decidua*, *Arabidopsis thaliana*, *Hordeum vulgare*, and *Spirodela polyrhiza* were grown with the supplementation of 30 μM pregnenolone or progesterone. DMSO was used as a mock treatment. Four days after treatment, plant material was harvested and amounts of 17α-reduced steroid were analyzed. Steroid extracts were prepared with tissues in direct contact with steroid-containing medium (complete organisms of *C. reinhardtii*, *P. patens* (protonemata) and *S. polyrhiza*, embryogenic cells of *L. decidua* and roots of *H. vulgare* and *A. thaliana*). Amounts of produced 17α-hydroxyprogesterone after treatment with pregnenolone or progesterone are shown. Values are given as mean ± SE ($n = 3$), while individual measurements are shown as black dots (c). Statistical analysis can be seen in Supporting Information Table S6.

plants contain androgens, but no progestogens, this raises the question whether an equivalent reaction is catalyzed in plant cells.

We tested 17α-hydroxylation in plants (Fig. 5) by treating model organisms with PR, PO, and analyzed 17α-OHPR and 17α-OHPO quantities (Fig. 5c). While all treated plants (except *L. decidua*) accumulated 17α-OHPO, only *C. reinhardtii* treated with PR accumulated 17α-OHPR (2.16 ± 1.07 ng mg^{-1} DW). 17α-OHPR is typically found in mammals. Therefore, steroidogenesis of *C. reinhardtii* appears to be more similar to mammalians than higher plants. Consequently, *C. reinhardtii* accumulated DHEA after feeding with 17α-OHPR (Table 5) due to 17,20-lyases converting 17α-reduced steroids. In land plants, this was seen in monocots only (Table 5). AD accumulation was found in terrestrial plants, except *L. decidua* and *S. polyrhiza* treated with 17α-OHPO (Table 5). Monocots showed the highest 17,20-lyase activity for 17α-OHPO. 17,20-lyase activity seems to limit plant steroidogenesis.

It is unknown whether PR and PO treatment enhances androgen amounts (DHEA or AD). DHEA and AD values were analyzed after PR and PO treatments (Table 6).

PR and PO treatments did not lead to significant DHEA or AD accumulation. Therefore, 17,20-aldolase activity (i.e. 17α-hydroxylation and 17,20-lyation) seems to be a limiting step in plant steroidogenesis.

3β-Hydroxysteroid dehydrogenase reactions (3β-HSDs)

In mammalian steroidogenesis, 3β-hydroxy-Δ5-steroids are converted into 3-oxo-Δ5-steroids by 3β-HSDs under NAD^+ consumption (e.g. the conversion of PR into PO, see Fig. 6). Enzymes with 3β-HSD activity were identified in plants (Finsterbusch *et al.*, 1999; Herl *et al.*, 2007; Munkert *et al.*, 2014) and catalyze 3-oxo-Δ5-steroids conversion into 3β-hydroxy-Δ5-steroids by consumption of NADH/ H^+.

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Table 5 17,20-lyase reactions in plants using pregnenolone, progesterone, 17α-hydroxypregnenolone, and 17α-hydroxyprogesterone as substrates.

DHEA values (in ng mg^{-1} DW) after 17α-hydroxypregnenolone treatment		
Species	DMSO	17α-hydroxypregnenolone treatment
C. reinhardtii	0.14 ± 0.02	0.2 ± 0.07
P. patens	0	0
L. decidua	0	0
A. thaliana	2 ± 0.80	1.2 ± 0.10
H. vulgare	0.1 ± 0.04	0.3 ± 0.10
S. polyrhiza	0	0.3 ± 0.10

Androstenedione values (in ng mg^{-1} DW) after 17α-hydroxyprogesterone treatment		
Species	DMSO	17α-hydroxyprogesterone treatment
C. reinhardtii	0	0
P. patens	0	0.1 ± 0.01
L. decidua	0	0
A. thaliana	0	0.5 ± 0.07
H. vulgare	0	5.7 ± 0.03
S. polyrhiza	0	5.8 ± 0.10

Plant species were cultivated on a medium supplemented with 30 μM 17α-hydroxypregnenolone, or 17α-hydroxyprogesterone. DMSO was used as a mock treatment. Four days later, the plant material was harvested and steroid profiles were analyzed. Steroid extracts were prepared with tissues in direct contact with steroid-containing medium (cells of *C. reinhardtii*, *P. patens* (protonemata), and *S. polyrhiza*, embryogenic cells of *L. decidua* and roots of *H. vulgare* and *A. thaliana*). Values are given as mean ± SE ($n = 3$). '0' indicates that the analyte was not detectable, that is, no or too low signal (S/N < 3) or absence of the respective qualifier signal. Statistical analysis can be seen in Supporting Information Table S6.

Table 6 Conversion of pregnenolone to DHEA and progesterone to androstenedione.

DHEA values (in ng mg^{-1} DW) after pregnenolone treatment		
Species	DMSO	Pregnenolone treatment
C. reinhardtii	0.003 ± 0.003	0.1 ± 0.001
P. patens	0	0
L. decidua	0	0
A. thaliana	1.7 ± 0.1	2 ± 0.02
H. vulgare	0.02 ± 0.007	0.004 ± 0.001
S. polyrhiza	0	0

Androstenedione values (in ng mg^{-1} DW) after progesterone treatment		
Species	DMSO	Progesterone treatment
C. reinhardtii	0	0
P. patens	0	0
L. decidua	0	0
A. thaliana	0	0
H. vulgare	0	0
S. polyrhiza	0	0

Plant species were cultivated on a medium supplemented with 30 μM pregnenolone or progesterone, while DMSO was used as a mock treatment. Four days after treatment cultures were harvested and steroid profiles were analyzed. The results of 17α-hydroxylase and 17,20-lyase reactions in plant steroidogenesis are shown. Steroid extracts were prepared with tissues in direct contact with steroid-containing medium (whole cell extract of *C. reinhardtii*, *P. patens* (protonemata), and *S. polyrhiza*), embryogenic cells of *L. decidua* and roots of *H. vulgare* and *A. thaliana*. Values are given as mean ± SE ($n = 3$). '0' indicates that the analyte was not detectable, that is, no or too low signal (S/N < 3) or absence of the respective qualifier signal. Statistical analysis can be seen in Supporting Information Table S6.

We fed *C. reinhardtii*, *P. patens*, *L. decidua*, *A. thaliana*, *H. vulgare*, and *S. polyrhiza* with 30 μM (final concentration) of Δ^5 steroids (PR, 17α-OHPR, DHEA) and Δ^4 steroids (PO, 17α-OHPO and AD). After 4 d, the plant material was harvested and steroid profiles analyzed.

All tested plants converted Δ^5 into Δ^4 steroids, while an accumulation of Δ^5 steroids in response to treatment with Δ^4 steroids could only be observed in *C. reinhardtii* and *A. thaliana* (Table 7). Comparing Δ^5 steroids conversions shows PR is the favored substrate of plant 3β-HSD.

17β-hydroxysteroid dehydrogenase reaction

Mammalian 17β-HSDs convert AD into TO (Fig. 7a). TO and DHT are widespread androgens in plants (Tables 2–4). We analyzed AD conversion into TO in plants (Fig. 7b) and the reverse reaction (Fig. 7c).

Treatment with AD and TO lead to, respectively, enhanced TO and AD values in all analyzed plants. TO levels were higher after AD application, compared with the reverse experiment. We assume that TO is produced from the intermediate AD. To test this hypothesis, we analyzed TO accumulation after treatment with PR, PO, 17α-OHPR, 17α-OHPO, or DHEA (Table 8).

Treatment with early androgens (e.g. AD in Fig. 6 and DHEA in Table 8) increased testosterone in all analyzed plants, while progestogen treatment was less effective. *H. vulgare* cultures treated with PR, PO, 17α-OHPR, or 17α-OHPO showed increased levels of testosterone, while 17α-reduced steroids induced low amounts of testosterone in *S. polyrhiza*. Testosterone values were higher in monocots when treated with 17α-OHPO compared with 17α-OHPR. 17,20-lyase activity seems to be higher in monocot species treated with 17α-OHPO (Table 4), indicating that 17,20-lyase activity limits plant steroidogenesis.

Steroid 5α-reductase reaction

Testosterone conversion into DHT by steroid 5α-reductases is the final step in human steroidogenesis. Human 5α-reductases also convert PO into DHP. The *A. thaliana DET2* gene encodes a functional steroid 5α-reductase, and steroid 5α-reductase products are found in *A. thaliana*. DHT was found in plant shoots (Tables 2–4) with values of 0.52 ± 0.01 ng mg^{-1} DW in *A. thaliana* shoots and 0.04 ± 0.01 ng mg^{-1} DW in roots. We identified DHP (1.20 ± 0.16 ng mg^{-1} DW) only in *A. thaliana* roots. We tested the conversion of PO and TO into DHP and

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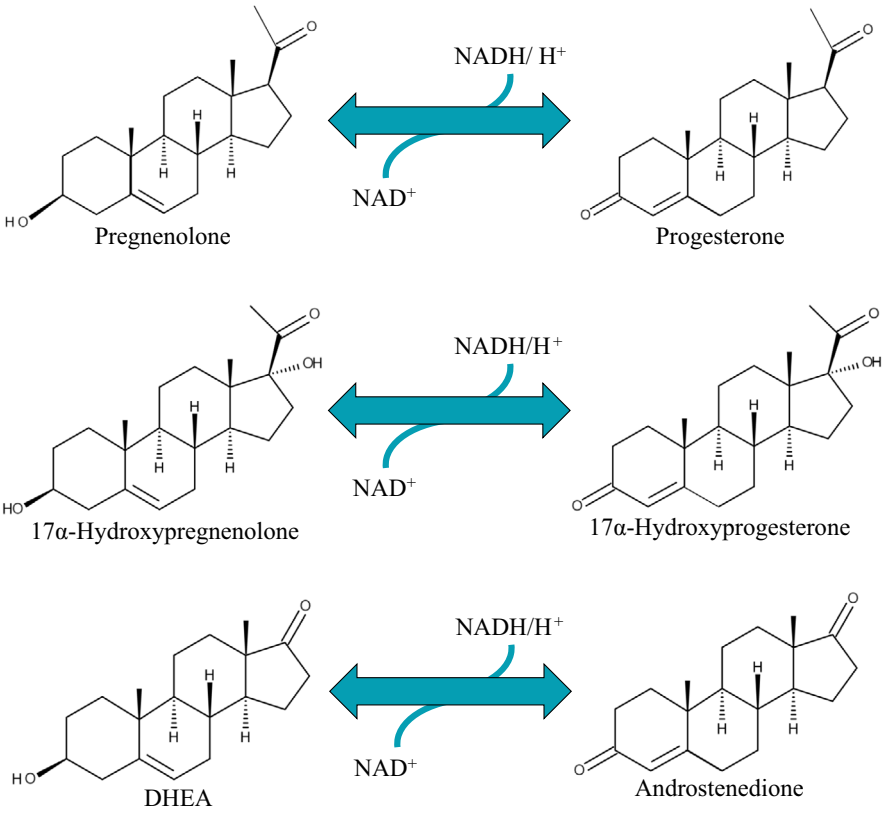

Fig. 6 3β-HSD reaction in steroid-treated plants. Reversible conversion Δ^5 steroids (PR, 17α-OHPR, DHEA) into Δ^4 (PO, 17α-OHPO, and A) by consumption of NAD^+.

DHT in *C. reinhardtii*, *P. patens*, *L. decidua*, *A. thaliana*, *H. vulgare*, and *S. polyrhiza*.

All plants accumulated DHP and DHT after PO and TO treatment (Fig. 8). Therefore, we assume that steroid 5α-reductases are highly conserved in plants. We tested whether DHP and DHT accumulate after treatment with steroids assumed to be the respective precursors (Table 9).

All plants converted progestogens into DHP, a 5α-reduced progestogen. Androgens were converted into 5α-reduced androgen DHT. 17α-OHPR and 17α-OHPO progestogens on the verge of androgen formation enhanced neither DHP nor DHT values. 5α-reduction of steroids is important in plant steroidogenesis, and DHT is abundant in photosynthetic tissues (Tables 2–4). The conversion of progestogens into androgens seems to be limited by 17,20-lyase activity.

Discussion

Estrogens were the first metabolites with steroid skeleton found in plants (Skarzynski, 1933). Until the 1960s, no further steroids were detected in plants (Heftmann, 1963). Nowadays, brassinosteroids, progestogens, and androgens are known steroids in plants.

To date, a systematic analysis of progestogen and androgen biosynthesis was missing. Our study closes this gap and sheds light on the evolutionary relationships of progestogen and androgen biosynthesis in plants.

Occurrence of progestogens and androgens in plants

Identification of progestogens started in the 1960s with PO in apple seeds (Gawienowski & Gibbs, 1968). Progestogens and androgens in nonseed plants and algae were not analyzed before, apart from *Equisetum hyemale* (*E. hyemale*) by Simons & Grinwich (1989). DHEA is present in the green algae *C. reinhardtii* and *C. vulgaris* (Table 2). DHEA was identified in all analyzed non-Spermatophyta, including mosses, club mosses, ferns, and Equisetidae (Table 1).

Androgen biosynthesis developed early during evolution. So, they are detectable in green algae, while no progestogens were identified in *C. reinhardtii* and *C. vulgaris* (Table 2). It is unknown whether progestogen precursors in green algae produce endogenous DHEA. In terrestrial nonseed plants, apart from *E. arvense*, progestogens, particularly PO, were also detected (Table 2). Moreover, Simons & Grinwich (1989) identified PO in the Equisetidae *E. hyemale*. We postulate that PO occurrence is linked to plants adapting to land. Photosynthetic organisms on land emit molecular oxygen (O_2) into the atmosphere. Consequently, land plants had to develop elaborate systems to prevent oxidative stress by reactive oxygen species (ROS). Exogenously applied PO induces ROS detoxification enzyme expression and reduces H_2O_2 formation during stress (Genisel *et al.*, 2013; Hao *et al.*, 2019). PO in plants potentially aid ROS detoxification. In humans, loss of PO causes decreased prostaglandin metabolism and loss of ROS protection (Evans & Salamonsen, 2012).

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Table 7 3β-hydroxysteroid dehydrogenase reactions in plants.

Progesterone values (in ng mg^{-1} DW) after pregnenolone treatment

Species	DMSO	PR treatment
C. reinhardtii	0	5690 ± 804
P. patens	0	1100 ± 6.87
L. decidua	0	9.7 ± 1.80
A. thaliana	0	380 ± 4.0
H. vulgare	3.2 ± 1.0	760 ± 234
S. polyrhiza	0	700 ± 45.8

Pregnenolone values (in ng mg^{-1} DW) after progesterone treatment

Species	DMSO	PO treatment
C. reinhardtii	0	5 ± 0.37
P. patens	0	0
L. decidua	0	0
A. thaliana	0	1 ± 0.16
H. vulgare	0	0
S. polyrhiza	0	0

17α-hydroxyprogesterone values (in ng mg^{-1} DW) after 17α-hydroxypregnenolone treatment

Species	DMSO	17α-OHPR treatment
C. reinhardtii	0	40 ± 4.4
P. patens	0	3 ± 1.1
L. decidua	0	0.3 ± 0.10
A. thaliana	0	44 ± 3.02
H. vulgare	0	10 ± 1.06
S. polyrhiza	0	90 ± 4.4

17α-hydroxypregnenolone values (in ng mg^{-1} DW) after 17α-hydroxyprogesterone treatment

Species	DMSO	17α-OHPO treatment
C. reinhardtii	0	0
P. patens	0	0
L. decidua	0	0
A. thaliana	0	0
H. vulgare	0	0
S. polyrhiza	0	0

Androstenedione values (in ng mg^{-1} DW) after DHEA treatment

Species	DMSO	DHEA treatment
C. reinhardtii	0	0.05 ± 0.01
P. patens	0	0.5 ± 0.11
L. decidua	0	0.3 ± 0.01
A. thaliana	0	1.4 ± 0.01
H. vulgare	0	0.02 ± 0.02
S. polyrhiza	0	0.3 ± 0.02

DHEA values (in ng mg^{-1} DW) after androstenedione treatment

Species	DMSO	AD treatment
C. reinhardtii	0.003 ± 0.003	0.03 ± 0.02
P. patens	0	0.004 ± 0.001
L. decidua	0	0

Table 7 (Continued)

DHEA values (in ng mg^{-1} DW) after androstenedione treatment

Species	DMSO	AD treatment
A. thaliana	0.02 ± 0.00	0.04 ± 0.00
H. vulgare	0.02 ± 0.003	0.3 ± 0.11
S. polyrhiza	0	0.02 ± 0.002

Plant species were cultivated on a medium supplemented with 30 μM pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, DHEA or androstenedione. DMSO was used as mock treatment. Four days after treatment, plant material was harvested and steroid profiles were analyzed. The table shows the results of the reversible 3β-hydroxysteroid dehydrogenase reaction involved in steroidogenesis. Steroid extracts were prepared from tissues in direct contact with steroid-containing medium (complete organisms of *C. reinhardtii*, *P. patens* (protonemata), and *S. polyrhiza*, embryogenic cells of *L. decidua*, and roots of *H. vulgare* and *A. thaliana*). Values are given as mean ± SE ($n = 3$). '0' indicates that the analyte was not detectable, that is, no or too low signal (S/N < 3) or absence of the respective qualifier signal. Statistical analysis can be seen in Supporting Information Table S6.

Progestogens and androgens in gymnosperms seem to be species-dependent (Table 2). Previous data (Simons & Grinwich, 1989) showed species-dependent steroid distribution in 128 plants. Unfortunately, the method used in this study detected only four steroids and did not differentiate between TO and DHT. We identified PO and PR in *P. pinea* and *C. revoluta* leaves. Carson *et al.* (2008) identified PO in needles of loblolly pine (*P. taeda*; 3.9 ng mg^{-1}). No androgens were found in *P. pinea* and *C. revoluta* leaves. Kolbah and colleagues identified androgens (e.g. AD and TO) in *P. silvestris* (Šaden-Krehula *et al.*, 1971) and *P. nigra* pollen (Šaden-Krehula *et al.*, 1979). Simons & Grinwich (1989) identified androgens in *Pinus banksiana* shoots. This supports an organ- or tissue-specific distribution of progestogens and androgens in plants as postulated by Simons & Grinwich (1989). Progestogens and androgens in angiosperms seem to be species-specific (Tables 3, 4). Simons & Grinwich (1989) demonstrated that PO was detectable in 80% of analyzed Spermatophyta, while TO and its derivatives were present in 70% of 128 analyzed Spermatophyta. Androgens were found in all analyzed plant seeds and the authors postulated organ-specific progestogen and androgen distribution.

We found that DHP was only detectable in *A. thaliana* roots but not in the shoots. In 1989, it was postulated that progestogens and androgens influence plant developmental processes (Simons & Grinwich, 1989). Our results indicate that monocot species have lower levels of progestogens and androgens than dicots, gymnosperms, and non-Spermatophyta (Table 3). Iino *et al.* (2007) reported PO levels comparable to our studies in shoots of *Oryza sativa*, while PO levels in *A. cepa* bulbs were very low. We attribute this to species-specific differences in angiosperms.

PO is an early precursor in 5β-cardenolide biosynthesis (Klein *et al.*, 2022). Our data indicate that 5β-cardenolide formation correlates with reduced *Brassicaceae* and *Plantaginaceae*

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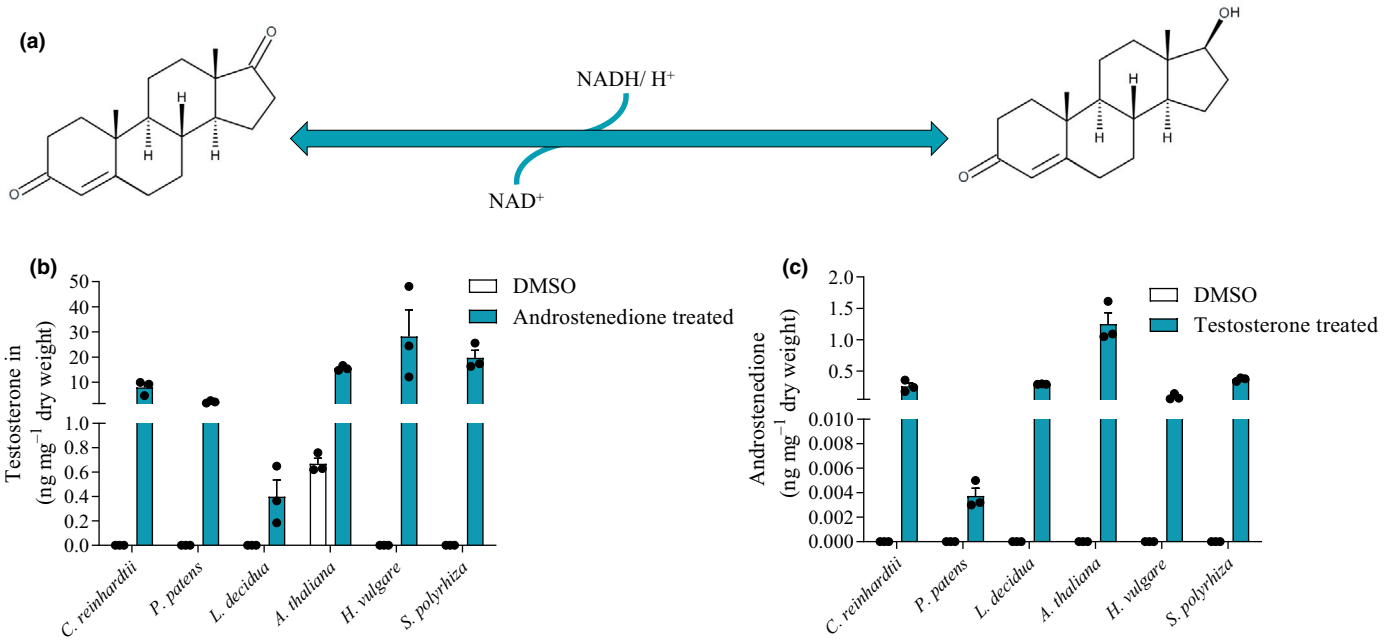

Fig. 7 17β-HSD reaction in steroidogenesis. The figure depicts the reversible conversion of androstenedione (AD) into testosterone (TO) by consumption of NAD^+ (a). To test whether plants catalyze 17β-HSD reactions of steroids, *Chlamydomonas reinhardtii*, *Physcomitrium patens*, *Larix decidua*, *Arabidopsis thaliana*, *Hordeum vulgare*, and *Spirodela polyrhiza* were cultivated on a medium supplemented with 30 μM androstenedione or testosterone, while DMSO was used as mock treatment. Four days after treatment, plant material was harvested and steroid profiles were analyzed. The figure shows the resulting testosterone contents in response to androstenedione treatment (b) and vice versa (c). Steroid extracts were prepared with tissues in direct contact with steroid-containing medium (complete organisms of *C. reinhardtii*, *P. patens* (protonemata), and *S. polyrhiza*, embryogenic cells of *L. decidua* and roots of *H. vulgare* and *A. thaliana*). Values are given as mean ± SE ($n = 3$), while individual measurements are shown as black dots. Statistical analysis can be seen in Supporting Information Table S6.

Table 8 Testosterone formation after treatment with different steroids.

TO values (in ng mg^{-1} DW) after steroid treatment						
Species	DMSO	PR	PO	17α-OHPR	17α-OHPO	DHEA
C. reinhardtii	0	0	0	0	0	1.7
P. patens	0	0	0	0	0	0.2
L. decidua	0	0	0	0	0	0.5
A. thaliana	0.7	1.70	0.1	0	0	72
H. vulgare	0	10	1.5	2.4	4.5	3.5
S. polyrhiza	0	0	0	0.7	0.8	4

C. reinhardtii, *P. patens*, *L. decidua*, *A. thaliana*, *H. vulgare*, and *S. polyrhiza* were cultivated on a medium supplemented with 30 μM pregnenolone (PR), progesterone (PO), 17α-hydroxypregnenolone (17α-OHPR), 17α-hydroxyprogesterone (17α-OHPO), or DHEA, while DMSO was used as mock treatment. Four d after treatment plant material was harvested and steroid profiles were analyzed. Steroid extracts were prepared with tissues in direct contact with steroid-containing medium (complete organisms of *C. reinhardtii*, *P. patens* (protonemata), and *S. polyrhiza*, embryogenic cells of *L. decidua* and roots of *H. vulgare* and *A. thaliana*). Values are given as mean ($n = 3$). '0' indicates that the analyte was not detectable, that is, no or too low signal (S/N < 3) or absence of the respective qualifier signal. Statistical analysis can be seen in Supporting Information Table S6.

progestogen levels. This might be due to enhanced consumption of progestogens in the form of PO by 5β-reduction catalyzed by steroid 5β-reductase/iridoid synthase-like enzymes (PRISEs). Exogenously applied PR and PO are partially converted by PRISEs in 5β-cardenolide-containing plants (Sauer *et al.*, 1967; Bennett *et al.*, 1968; Klein *et al.*, 2022). PRISE conversion of progesterone is essential for 5β-cardenolide formation and RNAi-mediated knockdown reduced 5β-cardenolides in *D. lanata* shoots (Klein *et al.*, 2022).

Progestogen and androgen distribution in plants has evolved early and remained conserved. This indicates their importance in plants.

Cholesterol as the sterol precursor for steroidogenesis

It is unclear which sterol is the plant steroidogenesis precursor. It was shown that treatment with radio-labeled CO (Bennett & Heftmann, 1966) and β-sitosterol (Bennett & Heftmann, 1965) led to labeled pregnenolone in plants. Mammalian CYP11A1, which catalyzes sterol conversion to PR, accepts a broad range of sterols as substrates, including desmosterol, campesterol, and β-sitosterol (Hartz *et al.*, 2021). CO is the primary sterol precursor for mammalian steroidogenesis. We compared CO and progestogen (PR and PO) levels and found no correlation. This could be caused by the conversion of different sterol precursors to PR, as previously reported for *D. lanata* CYP87A3 (Carroll *et al.*, 2022).

Another explanation could be the high cholesterol content of different plant organelles. Cholesterol distribution varies strongly among organelles (reviewed in Behrman & Gopalan, 2005). Cholesterol accounts for 1% of the total sterol contents of green

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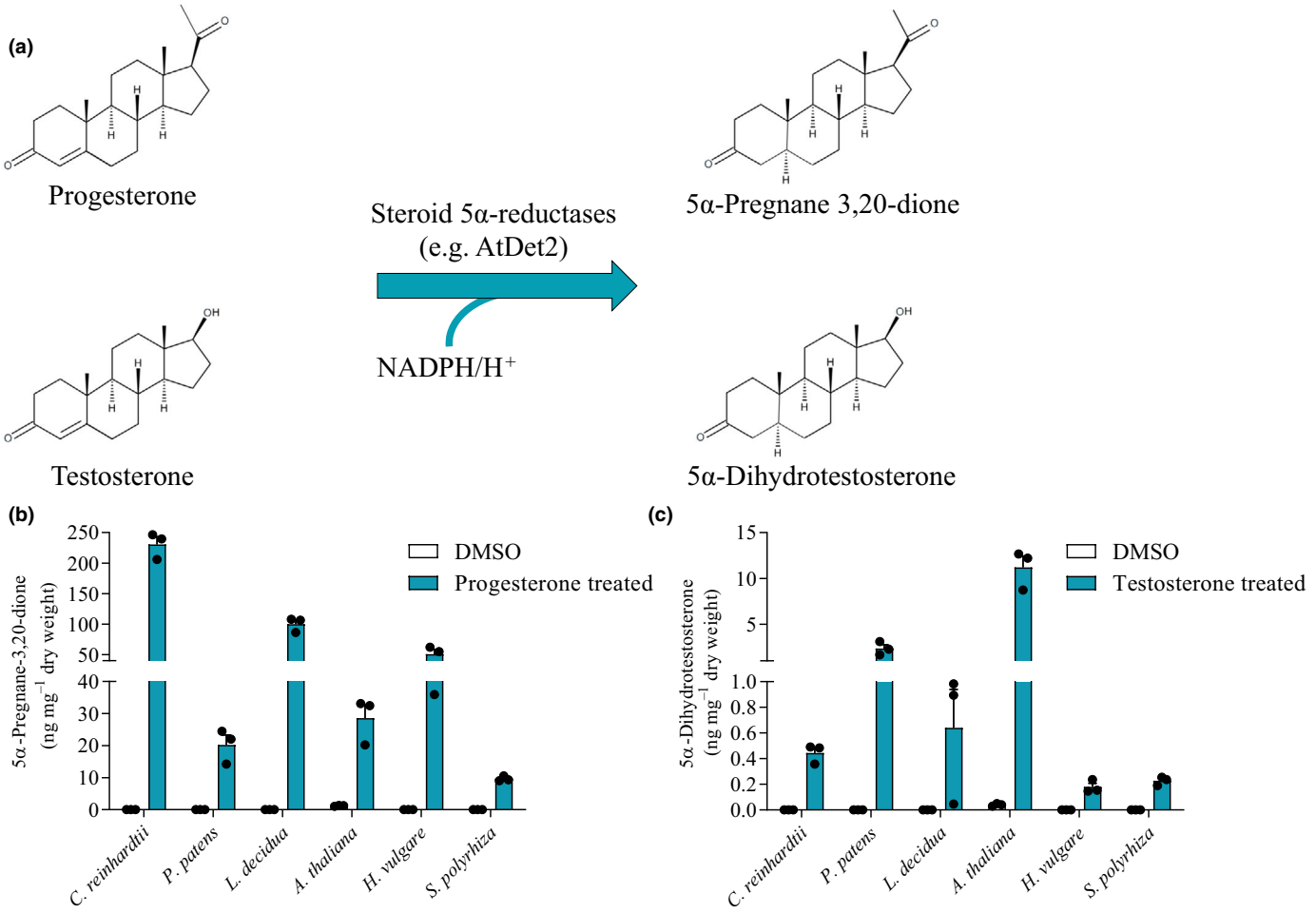

Fig. 8 5α-reductase reaction in steroid-treated plants. The graph depicts the reversible conversion of progesterone (PO) into 5α-pregnane-3,20-dione (DHP) and of testosterone (TO) into dihydrotestosterone (DHT) by consumption of NADPH/H^+ (a). *Chlamydomonas reinhardtii*, *Physcomitrium patens*, *Larix decidua*, *Arabidopsis thaliana*, *Hordeum vulgare*, and *Spirodela polyrhiza* were cultivated on a medium supplemented with 30 μM progesterone (b), or testosterone (c), while DMSO was used as mock treatment. Four days after treatment, plant material was harvested and steroid profiles were analyzed. The table shows the resulting DHP (b) or DHT (c) contents. Steroid extracts were prepared with tissues in direct contact with steroid-containing medium (complete organisms of *C. reinhardtii*, *P. patens* (protonemata), and *S. polyrhiza*, embryogenic cells of *L. decidua* and roots of *H. vulgare* and *A. thaliana*). Values are given as mean ± SE ($n = 3$), while individual measurements are shown as black dots. Statistical analysis can be seen in Supporting Information Table S6.

bean leaves while making up 24% of chloroplast sterols (Brandt & Benveniste, 1972). Maize nuclei sterols reported 22% cholesterol (Kemp & Mercer, 1968). This explains the lack of correlation between cholesterol and progestogen levels (Fig. 3; Table S1). Future studies elucidating subcellular localizations of sterol-steroid conversions in plants can refine the role of cholesterol in plant steroidogenesis.

We expressed a plant-codon-optimized CYP11A1 in *A. thaliana* (CO content: 59 μg g^{-1} DW) and *P. patens* (CO content: 205 μg g^{-1} DW). While transgenic protonemata of *P. patens* showed increases in PR, PO, and DHP (Fig. S5), this was not observed in *A. thaliana*. Bovine CYP11A1 expression in tomato and tobacco plants increased PR and PO levels (Shpakovski *et al.*, 2017). CO levels of tobacco leaves (*Nicotiana benthamiana*; 267 μg g^{-1} DW) are comparable to those of *P. patens*, indicating that CO is one sterol precursor of steroidogenesis in plants.

In mammals, the cleavage of sterols leading to PR is caused by three sequential monooxygenase reactions (Fig. 9). CO reduction to 22*R*-hydroxycholesterol and (22α,22*R*)-dihydroxycholesterol leads to C20-C22-bond cleavage, resulting in PR. CYP90B1 acts as a sterol monooxygenase catalyzing CO reduction at C22 in plants. A *D. lanata* enzyme catalyzing sterol conversion to PR was recently described (Carroll *et al.*, 2022).

Biotransformation of progestogens and androgens in plants

The wide distribution of progestogens and androgens in plants, and the existence of androgens (e.g. DHEA) in progestogen-free plants, raised the question of androgen biosynthesis. It was also questioned whether androgen biosynthesis depends on progestogen formation in plants and whether steroidogenesis was conserved during evolution.

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Table 9 Accumulation of 5α-reduced steroids after treatment with different steroids.

	DHP values (in ng mg^{-1} DW) after steroid treatment						
Species	DMSO	PR	17α-OHPR	17α-OHPO	DHEA	AD	TO
C. reinhardtii	0	40	0	0	0	0	0
P. patens	0	8	0	0	0	0	0
L. decidua	0	1	0	0	0	0	0
A. thaliana	1.20	11.7	0.3	0.9	1.8	0.01	2.18
H. vulgare	0	0.5	0	0	0	0	0
S. polyrhiza	0	4	0	0	0	0	0

	DHT values (in ng mg^{-1} DW) after steroid treatment						
Species	DMSO	PR	PO	17α-OHPR	17α-OHPO	AD	DHEA
C. reinhardtii	0	0	0	0.2	0.40	0.40	0.1
P. patens	0	0	0	0	0	0	0
L. decidua	0	0	0	0	0	0.40	0.3
A. thaliana	0.04 ± 0.01	0.05	0.07	0	0	5.6	11
H. vulgare	0	0	0	0	0	0.1	0.1
S. polyrhiza	0	0	0	0	0	0.3	0.1

C. reinhardtii, *P. patens*, *L. decidua*, *A. thaliana*, *H. vulgare*, and *S. polyrhiza* were cultivated on a medium supplemented with 30 μM PR, PO, 17α-OHPR, 17α-OHPO, DHEA or TO, while DMSO was used as mock treatment. Four days after treatment, plant material was harvested and steroid profiles were analyzed. The table shows the resulting DHP or DHT contents. Steroid extracts were prepared with tissues in direct contact with steroid-containing medium (complete organisms of *C. reinhardtii*, *P. patens* (protonemata), and *S. polyrhiza*, embryogenic cells of *L. decidua* and roots of *H. vulgare* and *A. thaliana*). Values are given as mean ($n = 3$). '0' indicates that the analyte was not detectable, that is, no or too low signal (S/N < 3) or absence of the respective qualifier signal. Statistical analysis can be seen in Supporting Information Table S6.

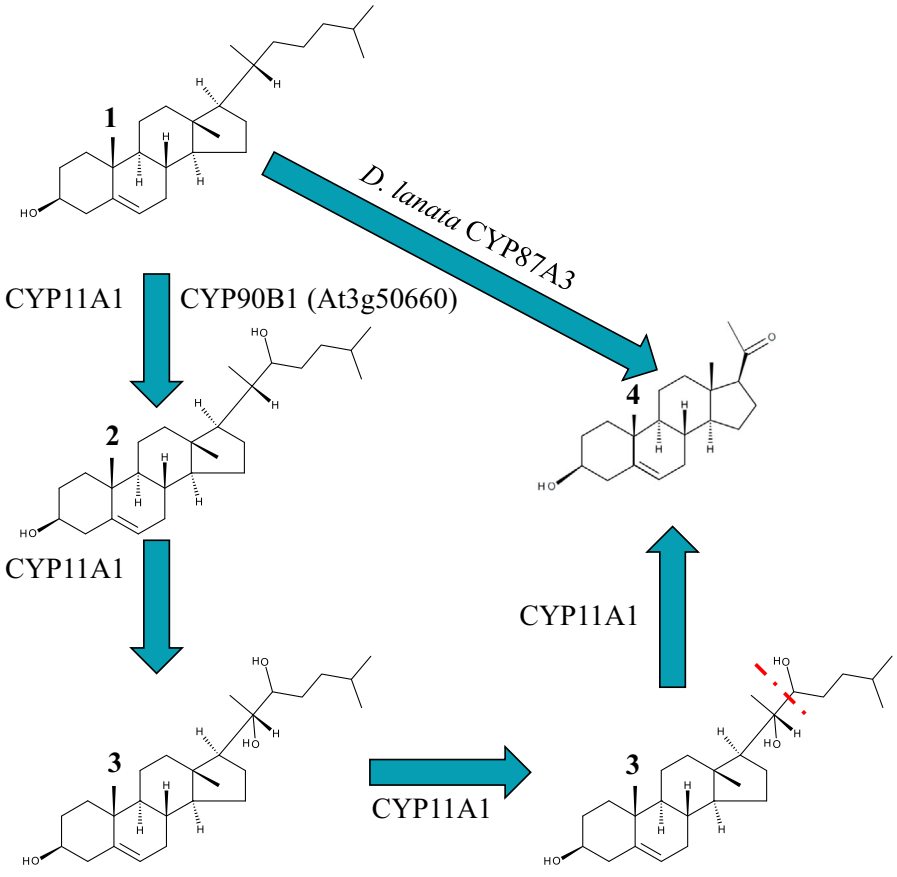

Fig. 9 Conversion of cholesterol into pregnenolone. The conversion of cholesterol (1) to pregnenolone (4) is caused by three sequential hydroxylation reactions in mammals. The progressive oxidation of cholesterol to (22*R*)-22-hydroxycholesterol (2) and (20*R*,22*R*)-20,22-dihydroxycholesterol (3) leads to cleavage of the bond between C20 and C22, which results in pregnenolone production (4). In *Arabidopsis thaliana*, CYP90B1 (At3g50660) can catalyze the reduction of cholesterol to (22)*R*-22-hydroxycholesterol. Moreover, a first plant sterol side chain cleavage enzyme was recently found in *D. lanata* (CYP87A3; Carroll *et al*., 2022). CYP87A3 catalyzes the conversion of cholesterol into pregnenolone, but the reaction mechanism is still unknown.

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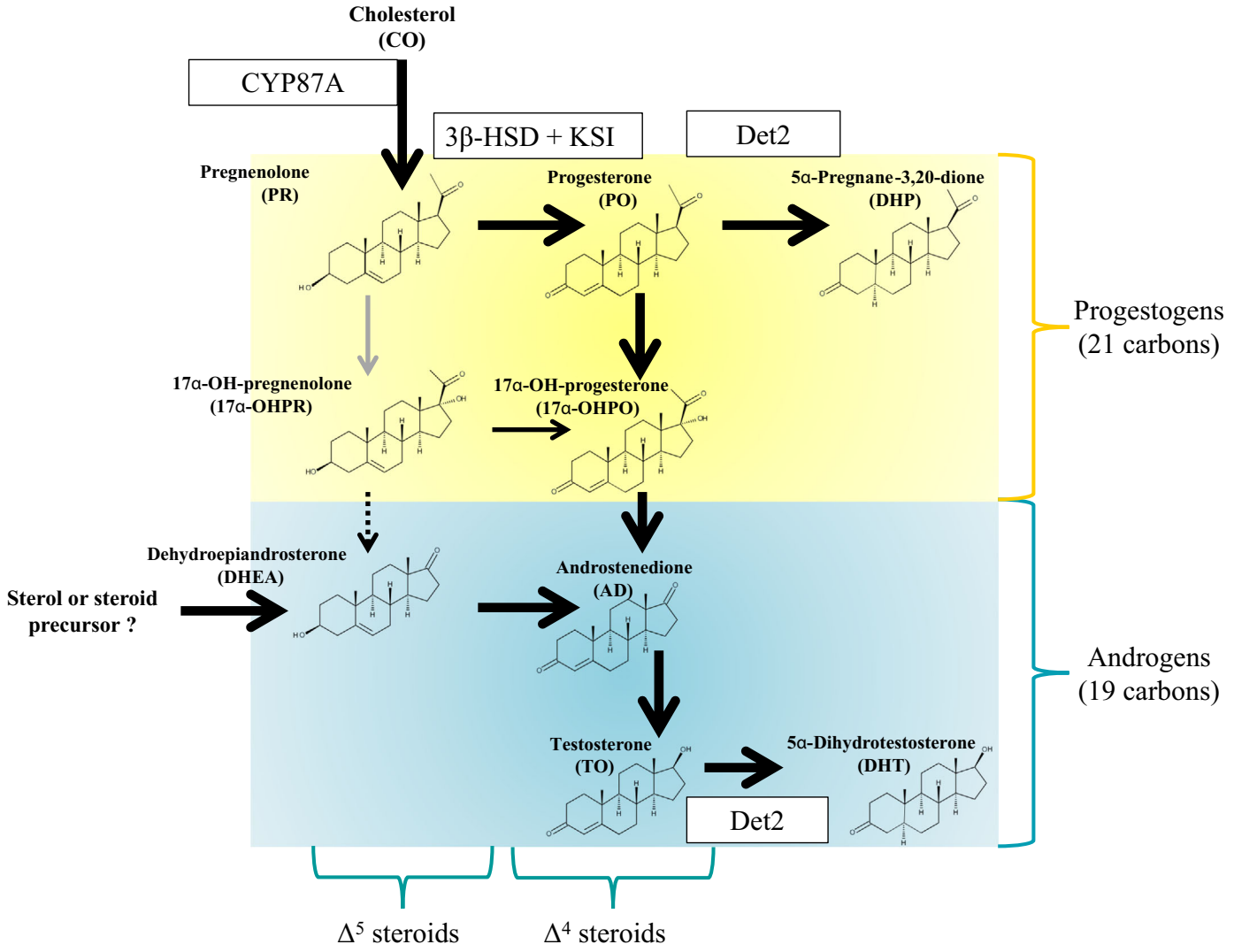

Fig. 10 Conversion of steroids in plants – a theory of plant steroidogenesis. Most reactions known from mammalian steroidogenesis are conserved in plants (bold arrows). The 17α-hydroxylation of pregnenolone (gray) was only found in *Chlamydomonas reinhardtii* suggesting a more mammalian-like steroidogenesis in this organism. 17,20-lyase of steroids, a reaction necessary for the conversion of progestogens into androgens, was more effective for 17α-hydroxyprogesterone. The conversion of 17α-hydroxypregnenolone (dashed arrow) to DHEA was detected only in monocots. This suggests that androgen biosynthesis in plants favors a route using the Δ^4 pathway.

To investigate the main pathways of steroidogenesis in plants, *C. reinhardtii*, *P. patens*, embryonic cells of *L. decidua*, *A. thaliana*, *H. vulgare*, and *S. polyrhiza* (Fig. 4) were treated with PR, PO, 17α-OHPR, 17α-OHPO, DHEA, AD, and TO. Many steroidogenesis reactions were conserved during plant evolution. *S. polyrhiza*, an aquatic monocot free of progestogens and androgens, catalyzes most of the analyzed reactions. All organisms converted PR into PO (Fig. 6; Table 7). For analyzed angiosperms, this reaction is the most effective 3β-hydroxysteroid dehydrogenation (Table 7). 3β-HSDs were already described in plants. The group of Kreis (Herl *et al.*, 2007; Munkert *et al.*, 2014) showed that a short-chain dehydrogenase (SDR) superfamily protein catalyzed pregnenolone conversion into isoprogesterone. Isoprogesterone is converted into progesterone by a Δ^5-3-ketosteroid isomerase (KSI). A KSI was purified from *D. lanata* protein extracts, but the amino acid sequence is still unknown (Meitinger *et al.*, 2015, 2016). Heterologously expressed 3β-HSD shows higher affinity to PR (K_m = 90 μM) as substrate in comparison with other tested steroids (Herl *et al.*, 2007). Finally, Witt (2008) showed that several SDR members of *A. thaliana*, including SDR5 and ABA2, can catalyze 3β-HSD reactions. The heterologously expressed enzymes *At*HSD1 (At2g47140) and *At*HSD2 (At2g47130) showed similar substrate affinities to PR (*At*HSD1: 50 μM; *At*HSD2: 43 μM). We showed conservation of PR to PO conversion among green algae and angiosperms. 3β-HSD activity can be detected in different plant organs and tissues, from moss protonema cells to embryonic cell cultures of the gymnosperm *L. decidua*. All plants catalyzed 3β-HSD reactions using 17α-OHPR or DHEA as substrates (Table 7). Witt (2008) showed that purified 3β-HSDs from *A. thaliana* catalyze 17β-HSD reactions. All analyzed plants convert AD into TO (Fig. 7). Our experiments showed that plants' 17β-HSD activity is not organ- or tissue-specific. The existence of both 3β- and 17β-HSD activity allowed the conversion of DHEA into TO (Table 8) in all analyzed plant species.

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The 17α-hydroxylase reaction of steroids initiates progestogen to androgen conversion. We tested whether plants accumulate 17α-OHPR or 17α-OHPO when treated with PR or PO (Fig. 5). All plants (except *L. decidua*) accumulated 17α-OHPO when treated with PR and PO with comparable efficiencies. However, 17α-OHPR, a human steroidogenesis intermediate (Hill *et al.*, 1999), could be detected only in *C. reinhardtii* treated with PR. This indicates a more mammalian-like conversion of steroids in *C. reinhardtii* compared with land plants, which is not surprising considering that many mammalian-like genes that are known in *C. reinhardtii*, such as selenoproteins and selenocysteines (Novoselov *et al.*, 2002) or animal-like cryptochromes (Zou *et al.*, 2017), could not be found in other plants.

17,20-Lyase reactions complete the formation of first androgens (DHEA and AD). We analyzed the conversion of 17α-OHPR or 17α-OHPO into DHEA or AD (Table 5) and found that land plants (except embryonic cells of *L. decidua*) accumulated AD after 17α-OHPO, while only monocots showed enhanced DHEA values after 17α-OHPR treatment. 17α-OHPR was only detected in 2 cardenolide-containing monocot species. PR and PO treatments did not enhance DHEA or AD values in analyzed plants. This indicates androgen biosynthesis is limited by 17,20-lyase reaction.

In humans, androgen biosynthesis is completed by steroid 5α-reduction of TO to DHT (the most active androgen). *AtDet2* encodes a functional steroid 5α-reductase and converts TO into DHT and PO to DHP (Li *et al.*, 1997). All organisms were able to convert progestogens into DHP and androgens into DHT (Fig. 8). Neither treatment with progestogens led to DHT formation, nor the reverse reaction took place (Table 9). This supports the hypothesis of independent progestogen and androgen formation in plants (Fig. 10).

Conclusions

(**1**) Progestogens and androgens are widespread steroids within the plant kingdom.
(**2**) DHEA, TO, and DHT are the most common steroids among the analyzed species.
(**3**) In green algae only DHEA was identified, while progestogens were identified only in the first land plants. This indicates a role of progestogens in plants adapting to land.
(**4**) The conversion of steroids seems to be conserved during plant evolution. This implies that steroidogenesis is under evolutionary pressure, and progestogens and androgens fulfill important functions in plants.
(**5**) All plants can convert Δ^5 into Δ^4 steroids. This is most effective for the conversion of PR into PO.
(**6**) In plants, converting Δ^4 progestogens into androgens is more effective than converting Δ^5 progestogens into androgens, indicating a preference for Δ^4 pathway to androgens.
(**7**) 17,20-Lyase activity and even the complete conversion of progestogens into androgens are most effective in the analyzed monocot species *H. vulgare* and *S. polyrhiza*. Therefore, the 17,20-lyase activity could be the limiting step in plant steroidogenesis.
(**8**) The existence of DHEA in a broad range of plants and the limited conversion of progestogens into androgens raises the question of whether plants contain progestogen-independent DHEA formation pathways.

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Competing interests

None declared.

Author contributions

RO, JAZZ, GK, BK, WV, FF and JK contributed to conceptualization; MR, FF, GS and JK contributed to method development instrumental analytics; M-JP, GS, FF, MN, VH-E and JK contributed to investigation; M-JP, GK, JZ, RO and JK contributed to writing – original draft preparation; RO, JAZZ, GK, BK, WV and JK contributed to writing – review and editing. All authors have read and agreed to the published version of the manuscript.

ORCID

Felix Feistel https://orcid.org/0000-0002-7085-4891
Vanessa Hermann-Ene https://orcid.org/0000-0001-8468-6369
Jan Klein https://orcid.org/0000-0003-2676-1505
Benedikt Kost https://orcid.org/0000-0003-2249-2555
Grit Kunert https://orcid.org/0000-0002-9969-8044
Maria Ntefidou https://orcid.org/0000-0003-0299-1242
Ralf Oelmüller https://orcid.org/0000-0002-3878-0044
Michael Reichelt https://orcid.org/0000-0002-6691-6500
Glendis Shiko https://orcid.org/0009-0006-9697-250X
Walter Vetter https://orcid.org/0000-0002-5592-4265
Julie A. Z. Zedler https://orcid.org/0000-0002-0462-8810

Data availability

Validation data for the established LC–MS/MS method for measurement of steroids in plants can be found at zenodo.org (Feistel *et al.*, 2023: doi: 10.5281/zenodo.7696597). Research data are contained within the article or Supporting Information.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Standard curves of steroid ions used as quantifiers.

Fig. S2 Standard curves of steroid ions used as qualifiers.

Fig. S3 Constructs for plant transformation.

Fig. S4 qPCR analysis of 35S:poCYP11A1 expressing *Arabidopsis thaliana* plants.

Fig. S5 qPCR analysis of Ubi:poCYP11A1 expressing protonemata of *Physcomitrium patens*.

Table S1 Cultivation media used for plant tissue culture and steroid biotransformation.

Table S2 Nucleic acid and amino acid sequence of the plant-optimized, human Cyp11A1 (poCyp11A1).

Table S3 Primer list. The primers used in this study can be found here in 5′–3′ orientation.

Table S4 Steroid solutions used for the validation of the UHPLC–MS-based quantification of progestogens and androgens in plants.

Table S5 Influence of different plant matrices on signal intensity (as peak area in counts) of nona-deuterated progesterone-D9 (PO-D9) quantifier ion.

Table S6 Statistical analysis of steroid conversion.

Table S7 Steroid and cholesterol contents in plants.

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