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The occurrence of progesterone 5β-reductase is not limited to the angiosperms: a functional gene was identified in *Picea sitchensis* and expressed in *Escherichia coli*

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Abstract

Background: Progesterone 5β-reductases (P5ßRs) catalyse one step in the stereospecific biosynthesis of cardenolides (potent repellents of herbivores and pharmaceutical stimulants of disordered heart muscle cells). They were originally discovered in the genus *Digitalis* and have since been frequently isolated from other angiosperms. Recombinant P5ßRs engineered in *Escherichia coli* host cells convert a broad spectrum of compounds *in vitro*, sometimes with higher efficiency than with progesterone. This observation suggests additional functions for cardenolide metabolism and promises future use in sustainable chemistry and biotechnology.

Methods: A tissue complementary DNA (cDNA) library was screened for orthologous P5ßRs. Candidates were subcloned into expression vectors and overexpressed in *E. coli* cells. The recombinant P5ßR protein was investigated for catalytic activity with several related substrates. Using spectrophotometric assays, the biochemical parameters of the enzyme were calculated. A 3D model was created and was compared to the previously published P5ßR structure of *Digitalis lanata* and other plant P5ßR models.

Results: Performing protein similarity searches in public databases and comparison of 3D protein structure models revealed four cDNA clones in a tissue library of *Picea sitchensis* (Bong.) Carrière putatively encoding P5ßRs. Succeeding with the expression of one clone in *E. coli*, the highly purified protein was unambiguously able to enantioselectively convert progesterone into 5ß-pregnane-3,20-dione. However, the catalytic activity to reduce the small molecule 2-cyclohexen-1-one was nearly 100 times faster. Methyl vinyl ketone was reduced similar to results from previously studied angiosperm resources.

Conclusions: (i) The low catalytic efficiency for progesterone conversion agrees with the fact that conifers have not been reported to accumulate cardenolides. This finding suggests that alternate metabolic processes occur whereby the newly detected enzymes could transform smaller molecules rather than large ones such as progesterone. (ii) An ancient P5ßR gene appears to have existed in the last common ancestor of seed plants approximately 300 million years ago. If the diversification of P5ßRs, including the currently detected homologous iridoid synthase activity, was related to stress encountered during the transition to growth on land, then investigation of P5ßRs from pteridophytes and bryophytes should improve our knowledge of this enzyme class and elucidate the direction of evolution.

Keywords: Progesterone 5β-reductase, Expression, Substrate specificity, Pinaceae

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Background

Cardenolides are known to repel herbivores and are traditionally used in human medicine as a heart stimulant (Agrawal et al. 2012). They are a group of glycosylated steroidal compounds that occur in numerous families of angiosperms (Luckner and Wichtl 2000) and are frequently detected in dicotyledonous species (reviewed by Kreis and Müller-Uri 2010), e.g. the rosids (Crossosomatales; Myrtales; Celastrales; Malpighiales; Fabales; Rosales; Brassicales; Malvales) or the asterids (Gentianales; Lamiales; Solanales; Asterales). Cardenolides have also been reported from derived monocots (Poales; Asparagales; Liliales) as well as from ancestral eudicots (Ranunculales). This distribution suggests that cardenolides arise from genes that were already present during the early diversification of angiosperms (Krenn and Kopp 1998; Luckner and Wichtl 2000). Tarrio et al. (2011) claimed that they were probably spread by horizontal gene transfer between protobacteria and plant cells. On the basis of genomic evidence from pines (Gavidia et al. 2007), we assume that homologous genes existed in plant genomes prior to the evolutionary split of angiosperms and gymnosperms. However, this hypothesis must be regarded as unproved until it has been verified by acceptable enzymatic evidence.

Progesterone 5ß-reductase (P5ßR) controls chiral cardenolide biosynthesis because it reduces progesterone via hydride transfer and protonation exclusively to 5ß-pregnane-3,20-dione (Fig. 1; for the detailed mechanism of this stereo-selective 1–4 hydrogen addition, see Thorn et al. 2008), whereas no 5α-pregnane-3,20-dione has been found (Gärtner et al. 1990). Related enzymes have been detected in other plant species that do not accumulate cardenolides, following the initial purification of P5ßR activity from *Digitalis purpurea* L. by Gärtner et al. 1994. However, the identification of the resulting biochemical products remains a challenge (Herl et al. 2006, 2009; Gavidia et al. 2007; Bauer et al. 2010; Perez-Bermudez et al. 2010; Munkert et al. 2011, 2015a, 2015; Ernst et al. 2015).

All P5ßR enzymes isolated from plants belong to the short-chain dehydrogenase/reductase (SDR) superfamily (Kavanagh et al. 2008). They contain three highly conserved N-terminal protein motifs (I to III) responsible for binding the co-substrate NADPH. Because other standard SDR motifs are missing, plant P5ßR proteins represent a distinct SDR class (Thorn et al. 2008).

Five additional conserved amino acid motifs (IV to VIII), positioned towards the C-terminal protein half, have been discovered by sequence alignments, 3D modelling, and site-directed mutagenesis. These motifs play an important role in the molecular architecture including substrate binding and catalysis (Gavidia et al. 2007; Thorn et al. 2008; Perez-Bermudez et al. 2010; Bauer et al. 2010, 2012).

It is noteworthy that the expression of some P5ßR genes by plants is enhanced following wounding or in response to other stresses, such as increased mannitol concentration (Yang et al. 1997). However, P5ßR has been constitutively expressed at basal levels in all plant organs examined (Herl et al. 2009; Munkert et al. 2011). This pattern of expression can result from P5ßR encoding gene duplications, which gives rise to different regulated genes. Such an effect has been recently observed in *Catharanthus roseus* (L.) G. Don and *Medicago truncatula* Gaertn. (Munkert et al. 2015). In addition to single-stress defence reactions, it is possible that there is a fundamental role for P5ßR enzymes during plant development because an *Arabidopsis thaliana* (L.) Heynh. mutant has an abnormal leaf vein pattern (*VEP*) (Jun et al. 2002).

P5ßR enzymes are also promising tools for carrying out biotransformation (a current approach has been described by Durchschein et al. 2012) because they reduce *in vitro* a broad variety of molecules bearing an activated C=C double bond, such as monocyclic enones (e.g. 2-cyclohexen-1-one, as shown in Fig. 1) or acyclic enoate esters (e.g. ethyl acrylate). In some cases, these reactions occur with higher efficiencies

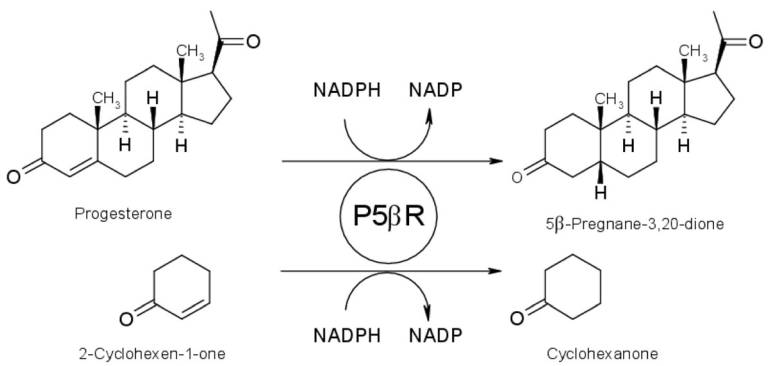

Fig. 1 Scheme of P5ßR reactions using progesterone or 2-cyclohexen-1-one as a substrate and NADPH as the co-substrate

than for progesterone (Burda et al. 2009; Reß et al. 2015).

This study identified P5ßR enzymes in conifer tree species, where such proteins have not previously been detected unambiguously. Initial testing of substrate specificity and catalytic efficiency was also undertaken.

Methods

Protein similarity searches and in silico analyses

Current releases of non-redundant databases were screened by the protein blast algorithm operating at the public NCBI server (www.ncbi.nlm.nih.gov/Blastp) to search for P5ßR sequences in conifers. For this purpose, the P5ßR sequence (AIF73578.1) from *Digitalis lanata* Ehrh. was used as a query target. A multiple alignment was carried out by the ClustalW software package maintained on a local computer (Larkin et al. 2007) in order to inspect the conifer sequences for the presence of the conserved protein motifs characterizing plant P5ßR proteins. Molecular weights for the native conifer proteins and for the recombinant hybrid proteins were estimated by a peptide mass calculator software (www.peptidesynthetics.co.uk). Alpha-helical protein regions and β-sheets were identified by current computational methods (www.swissmodel.expasy.org).

Modelling 3D protein structures

SWISS-MODEL (Kiefer et al. 2009), a fully automated software package running on a public server (www.swissmodel.expasy.org), was employed to compute the three-dimensional structure of protein ABK24388.1. The previously determined crystal structure PDB ID: 2V6G (Thorn et al. 2008) of a recombinant P5ßR protein from *D. lanata* in a complex with the co-substrate NADPH served as the starting structure to make the model. Progesterone was placed manually into the active protein site. A stereo representation was drawn using the PyMOL Molecular Graphics System programme (Version 1.5.0.4 Schrödinger, LLC). This procedure was also used to calculate and draw the three-dimensional protein structures of orthologous P5ßR sequences from *A. thaliana* (AAL32529.1) and *Erysimum crepidifolium* Rchb. (ADG56544.1), respectively.

DNA cloning into an *Escherichia coli* host vector

We designed the forward primer 5′-ggccat*ggatcc*tggtattcctggtggacaggttcc-3′ and the reverse primer 5'-ggccat*aagctt*ctaaggaacgatattagatgacctg-3′ (produced by biomers.net GmbH, Ulm, Germany) for a PCR-based amplification (5 min 94 °C; 35× [30 s 94 °C, 45 s 60 °C, 2 min 72 °C]; 7 min 72 °C) of the plasmid clone WS02727_DO9 (provided by J. Bohlmann, Vancouver, Canada), which contains a full-length complementary DNA (cDNA) sequence encoding protein ABK24388.1 from *Picea sitchensis* (Bong.) Carrière (Sitka spruce) (Ralph et al. 2008). The underlined nucleotides did not anneal with the cDNA but created a unique *Bam*HI site and a unique *Hind*III site, respectively, as indicated by the italicised letters. The 1.2-kb PCR product was digested with *Bam*HI and *Hind*III and inserted into the expression plasmid pET28 M-SUMO3-GFP (provided by H. Besir, EMBL Protein Expression & Purification Core Facility, Heidelberg, Germany), which had been previously cut with *Bam*HI and *Hind*III to replace the EGFP reporter gene by the PCR product. All cloning steps including transformation (see below) were carried out as described by Sambrook and Russell (2001). The enzymes were obtained from Thermo Scientific Inc. (Waltham, MA). The resulting plasmid (pET28 M-SUMO3-ABK24388.1) expresses a hybrid protein under the control of the T7 promoter with an N-terminus of six histidine residues fused in-frame with the ubiquitin-related SUMO protein as well as the spruce protein. This N-terminal modification enhances the solubility of the recombinant protein in *E. coli* (Butt et al. 2005) and aids in its chromatographic separation (see below).

Transformed *E. coli* of strain BL21DE3/pLys (Promega, Mannheim, Germany) were selected on Luria-Bertani (LB) agar medium containing kanamycin (20 mg/L) and chloramphenicol (10 mg/L). The plasmid DNA was analysed by digestion with restriction enzymes. A bacterial clone harbouring the plasmid pET28 M-SUMO3-ABK24388.1 was examined by the commercial LIGHTrun sequencing service (GATC Biotech, Köln, Germany) to confirm the correctness of the spruce nucleotide sequence.

Recombinant protein production and purification

Escherichia coli strain BL21DE3/pLysS, harbouring the plasmid pET28 M-SUMO3-ABK24388.1, was grown at 30 °C under rigorous shaking (250 rpm) in 1 to 2 L of LB medium supplemented with kanamycin (20 mg/L) and chloramphenicol (10 mg/L). At an OD_{600} of 0.8, IPTG was added to a final concentration of 1 mM. The culture was then incubated for 8 h. Cells were harvested by centrifugation (8.000×*g*, 30 min, 4 °C) and were suspended in 20 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) containing a "complete" protease inhibitor cocktail as advised by the manufacturer (Roche Diagnostics, Mannheim, Germany). For cell disruption, a French-Press (Aminco, Sandy, UT) was used under internal pressure of 20.000 psi. Ni-NTA affinity chromatography was carried out using a standard procedure (Qiagen GmbH, Hilden, Germany). Eluted protein was mixed with 1/10 volume of protease His6-SenP2 (125 ng/mL) previously purified from the strain *E. coli* BL21DE3/pLysS/pETM11-SenP2 (provided by H. Besir, EMBL Protein Expression & Purification Core Facility, Heidelberg). The mixture was maintained for 30 min at

37 °C, then tenfold diluted with 20 mM Tris/HCl (pH 8.0), and again subjected to Ni-NTA affinity chromatography. The flow-through was loaded onto an anion-exchange chromatography column (Resource Q, GE Healthcare Europe GmbH, Freiburg, Germany), equilibrated with 20 mM Tris/HCl (pH 8.0) at a flow rate of 1 mL/min using an Äkta Purifier FPLC device (GE Healthcare). The protein was finally eluted over 20 column volumes by means of a linear salt gradient (0 to 500 mM NaCl) in 20 mM Tris/HCl (pH 8.0). Fractions of the major protein peak were analysed by SDS-PAGE. Protein concentrations were estimated according to Bradford (1976).

Enzymatic tests, including product identification

Enzymes, substrates, co-substrates, and reference substances were provided by Sigma-Aldrich GmbH (Taufkirchen, Germany), except the recombinant P5ßR proteins produced by Munkert et al. (2011), Bauer et al. (2012), or reported here. Solvents were purchased from Carl Roth GmbH (Karlsruhe, Germany). Successful enzymatic conversion of progesterone (0.3 mM) to 5ß-pregnane-3,20-dione was achieved at 40 °C in samples containing 0.2 mg/mL recombinant protein and a NADPH regenerating system, consisting of 6.4 mM NADP, 32.1 mM glucose-6-phosphate, and 42 nkat glucose-6-phosphate dehydrogenase as described by Herl et al. (2009). Heat-inactivated samples (10 min, 100 °C) served as controls. After 12 h, enzymatic reactions were terminated by the addition of 1000 µL dichloromethane. After evaporation, the dry residue was dissolved in 50 µL methanol. To perform thin-layer chromatography (TLC), up to 20 µL was spotted onto silica gel $60F_{254}$ plates (VWR International, Radnor, PA) and run by using an 80:18:2 (*v*/*v*) mixture of dichloromethane, methanol, and water. The product formation was followed by Jensen-Kny detection (Jork 1990). The stereo-selective conversion of progesterone was examined from the remaining part after the evaporation of methanol and dissolving the residue again in 100 µL dichloromethane. A volume of 3 µL of this solution was used in gas chromatography-mass spectrometry (GC-MS) as described by Rudolph et al. (2014). To identify enzymatic products by TLC as well as GC-MS, the provided reference substances 5ß-pregnane-3,20-dione, 5α-pregnane-3,20-dione, and progesterone were tested alone.

To monitor the velocity of the reaction (k_{cat}) during 200 s at 40 °C and to identify substrate concentrations associated with half-maximal velocity (K_M), conversion of NADPH (0.4 mM) to NADP was recorded by a photometer (340 nm) in the presence of 0.05 to 0.4 mM substrate (progesterone, 2-cyclohexen-1-one, or methyl vinyl ketone). Kinetic parameters were obtained from the Shimadzu UV Probe software package.

Results and discussion

Protein alignment data

We scanned the protein databases to search for P5ßR sequences amongst conifer species by a computational similarity search and detected a few tentatively identified tissue library cDNA clones that encoded 54 to 55 % identical amino acids with the angiosperm target query. The identity of the corresponding proteins (ABK24243.1, ABK24388.1, ABR16676.1, and ABR16709.1 of *P. sitchensis*) ranged from 85 to 97 %, with ABK24243.1 and ABR16676.1 producing the best match. The four sequences shared eight conserved motifs characteristic for plant P5ßRs (Thorn et al. 2008; Perez-Bermudez et al. 2010). These motifs differ only slightly in comparison to the query. Motifs I, II, VI, VII, and VIII have one amino acid exchange each, motifs III and V show two individual variable positions, and motif IV has three amino acid replacements, as shown in Table 1. The alignment data suggest the existence of a homogeneous multi-gene family within the genome of Sitka spruce because the conifer proteins all encode 399 amino acids (one full-length sequence is shown in Fig. 2) and their individual molecular masses (45.3–45.4 kDa) range in size typical for plant P5ßRs. Such a family could provide *in planta* an enzymatic reduction of progesterone and related substrates. Small multi-gene families encoding P5ßR activities have been reported earlier for angiosperm species (Gavidia et al. 2007; Munkert et al. 2015a, 2015). We assume that an ancient gene was already present in the last common ancestor of gymnosperms and angiosperms approximately 300 million years ago, when the Coniferales appeared during the era of late Pennsylvanian era (Savard et al. 1994).

Gavidia et al. (2007) previously identified two pine genes in public databases using a similarity search programme (tblastn) and a P5ßR sequence from *D. lanata*. The authors used both entries to construct an unrooted phylogenetic tree with other plant species indicating poor bootstrap support (51 %) of the pine clade. We were unable to find either sequence again (their individual gene annotation numbers were not submitted) but acknowledge a striking similarity between spruce and pine with reference to both short diagnostic protein motifs.

3D structural protein modelling data

A possible 3D structure was calculated for protein ABK24388.1 to provide proof of the sequence similarities. Our model (Fig. 3a) shows an N-terminal double Rossman fold responsible for co-substrate binding, which corresponds to the stretch of repeated ß-sheet

Table 1 *Picea* protein motifs (I–VIII) compared to the consensus P5βR sequence and a sequence from *Digitalis lanata* (*DlP5ßR*)

	DlP5βR	*Picea sitchensis*	*Picea sitchensis*
Acc. Nr./consensus[a]	AIF73578.1	ABK24388.1	ABK24243.1
			ABK16676.1
			ABK16709.1
Motif I/GxxGxxG	GVTG**I**IGN	GVTGIVGN	GVTGIVGN
Motif II/GxxRR	GV**S**RR	GVARR	GVARR
Motif III/Dhx[cp]	D**IS**D	DVLD	DVLD
Motif IV/DhTHhFYYpW[hp]	DVTH**V**F**Y**V**T**W	DVTHLFWVVW	DVTHLFWVVW
Motif V/TgxKxYhG[hp]	TG**R**KHY**M**GP	TG**G**KHYLGP	TGAKHYLGP
Motif VI/NFYYxxED	NFYY**D**LED	NFYYTLED	NFYYTLED
Motif VII/Wp[VI]HPR	WS**V**HRP	WSIHRP	WSIHRP
Motif VIII/D[hp]DlhA[ED]	DA**D**LIAE	DAELIAE	DAELIAE

Amino acid variations amongst *Picea* proteins and to *DlP5βR* are indicated in bold. In the motifs, "c" denotes a charged residue, "h" a hydrophobic residue, "p" a polar residue, and "x" any possible residue
[a]Perez-Bermudez et al. 2010

and α-helix structural elements (Fig. 2) and is in agreement with data from other investigators (Thorn et al. 2008; Bauer et al. 2010, 2012). This region is connected to the hydrophobic substrate binding C-terminal domain positioned in the upper part of the model, which exclusively harbours the clustered helical amino acid stretches depicted in Fig. 2. A one-by-one comparison of the P5ßR protein models between *P. sitchensis* and two angiosperm species (Fig. 3b, c) revealed no striking differences.

Recombinant protein purification data

Escherichia coli was transformed with the plasmid pET28 M-SUMO3-ABK24388.1 to provide recombinant spruce protein for enzymatic investigations. A faint band at the expected 66.7-kDa molecular weight was visible by SDS-PAGE (Fig. 4, lane 2; lane 1 presents size markers) indicating low production of the recombinant protein within the host cells. Using the N-terminal 6× His tag, this protein was successfully enriched by a Ni-NTA chromatography and appeared afterwards as the most prominent band (Fig. 4, lane 3). Cleaving the N-terminus tag with SenP protease and repeated chromatography (Fig. 4, lanes 4 to 6) lead to the isolation of a single recombinant protein band of 45.2 kDa. This pure recombinant protein lacks the first methionine residue in comparison to the native spruce polypeptide ABK24388.1 due to the cloning procedure and the protease cleavage. Approximately 10 mg recombinant spruce protein was obtained from 2 L bacterial cultures.

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                                                    Motif I
MSSFSWWAGSLGHGVRKFETKGESSEDKSEKQQGVALVI GVTGIVGN SLVEILPLSDTPG
                                          sssssss   hhhhhhhhhh
          Motif II                  Motif III        Motif IV
GPWKIY GVARR PKPDWSPDTSVEYIQC DVLD RELTLEKISPLKD VTHLFWVVW VNRETEE
     ssssss                 sssss   hhhhhhhhh   ssssss          hh
                                    Motif V
QNCEDNGRMLSNVLDALLPNAENLQHICLQ TGAKHYLGP FDAVAGNRYFQPHEAPYHEEL
hhhhhhhhhhhhhhhhhh          sssssssshhhh
          Motif VI              Motif VII
PRLPVP NFYYTLED IVFEAAKKKDGLT WSIHRP SFIFGFSPWSLMNIVGTLCVYAAICKH
          hhhhhhhhhhhhhh      sssssss sss            hhhhhhhhhhhhhh
                          Motif VIII
EGLPFKYPGNRITWEQFVDIS DAELIAE QEIWAATYPHAKNQAFNCSNGDVLKWKRLWGI
h          hhhhh      ssshhhhhhhhhhh          sssss          hhhhhhh
IAEKFDLEPLPYKGEGFSLAEAMKDKGPVWDALVRENKLHPTKIEEVGNWWFAEFTLNLP
hhhhh              hhhhh     hhhhhhhhhh                hhhhhhhh
QEMINSMNKSKEYGFFGFRNTETSLGQWIDKMKASNAVP
          hhhhhh          hhhhhhhhhhhhhhh
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Fig. 2 Protein sequence of P5ßR from *P. sitchensis* (ABK24388.1). The conserved motifs (I to VIII) were printed in *bold* according to Perez-Bermudez et al. (2010). Letters below the sequence symbolize α-helices (*h*) and β-sheet-like structural elements (*s*)

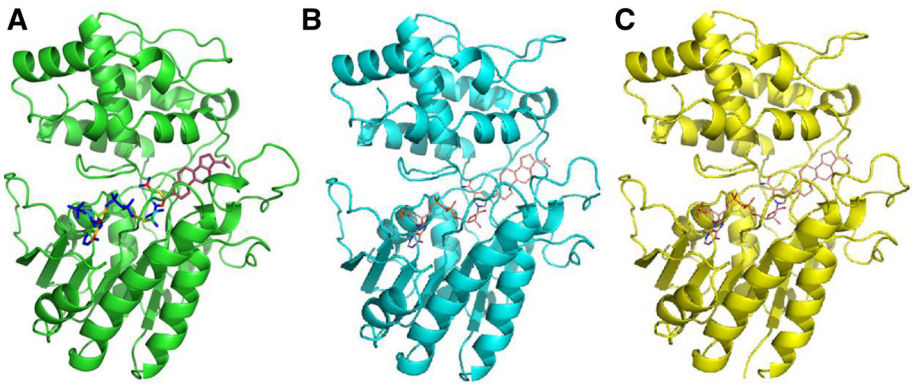

Fig. 3 Ribbon graphics representing the 3D structural protein data calculated for orthologous P5ßR enzymes from different species. **a** Protein ABK24388.1 from *P. sitchensis*. **b** Protein AAL32529.1 from *A. thaliana*. **c** Protein ADG56544.1 from *E. crepidifolium*

Enzymatic data

The highly purified recombinant spruce protein was able to convert progesterone into 5ß-pregnane-3,20-dione, indicating a genuine P5ßR enzyme activity, as shown by TLC analysis (Fig. 5) and analysis of the authentic sample by GC-MS (Fig. 6). This stereo-selective reaction occurred more slowly than with either of the recombinant angiosperm enzymes tested, which produced greater amounts of 5ß-pregnane-3,20-dione under the same conditions (Fig. 5b, see lanes 1 and 2 vs. lane 4). However, 5α-pregnane-3,20-dione was not formed by any of the enzymes studied and the conditions of the assays were always kept constant.

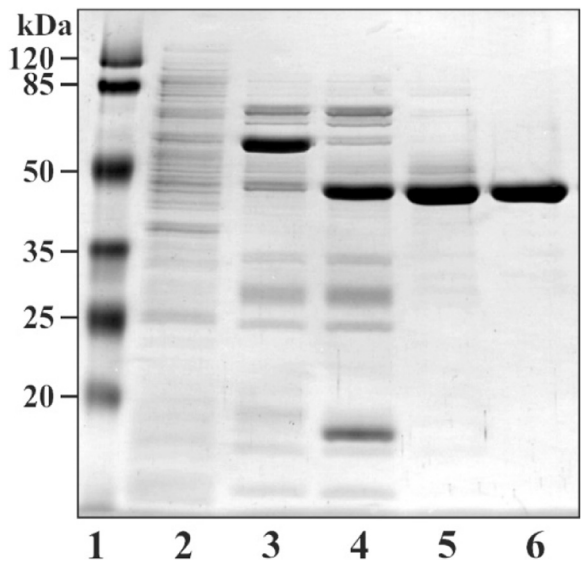

Fig. 4 Purification steps to isolate recombinant P5βR protein ABK24388.1 of *P. sitchensis* from *E. coli* host cells using an N-terminal 6× His tag SUMO protein fusion. Five micrograms of total protein per lane was analysed by a 12 % SDS-PAGE. *Lane 1*: Standard proteins of indicated molecular weights (Thermo #26612, Thermo Scientific, Darmstadt, Germany). *Lane 2*: Cell-free protein extract of *E. coli* BL21DE3/pLysS/ pET28 M-SUMO3-ABK24388.1 prepared 8 h after the addition of IPTG. *Lane 3*: Protein fraction eluted from the first Ni-NTA affinity column. *Lane 4*: Protein fraction eluted from the first Ni-NTA affinity column following incubation with SenP2 protease. *Lane 5*: Flow-through of the second Ni-NTA affinity column. *Lane 6*: Collected major peak fractions obtained after anion-exchange chromatography on a Resource Q column

Exact kinetic values were determined by a photometrical assay measuring the conversion of the co-substrate NADPH into NADP. For the recombinant spruce enzyme, the data (Table 2) reveal a low catalytic efficiency (k_{cat}/K_M) for the reduction of progesterone compared to recombinant angiosperm proteins studied by other investigators. This difference may be because only 11 out of the 17 conserved amino acid residues that are required for rapid catalytic action are present (Bauer et al. 2010; Petersen et al. 2015). The low catalytic efficiency associated with progesterone conversion increased 88.9 times when 2-cyclohexen-1-one was assessed. This molecule is smaller than progesterone and probably is a better fit for the catalytic site.

Enzymes are known to contain single residues controlling product building rates and functional evolution as analysed by Li et al. (2013). Tyrosine-156 (occurring in spruce as well), asparagine-205, and serine-248 were originally identified as hotspots responsible for the low catalytic efficiency reported for a recombinant P5ßR enzyme from *D. lanata*. Its catalytic performance was significantly improved *in vitro* after substituting asparagine in position 205 by methionine or alanine (Bauer et al. 2012).

The slow enzymatic reaction for progesterone in contrast to 2-cyclohexen-1-one agrees with the fact that spruce species have not been reported to accumulate cardenolides. This raises a question concerning a possible *in planta* function of the conifer proteins, which correspond in structure and origin to the P5ßR proteins

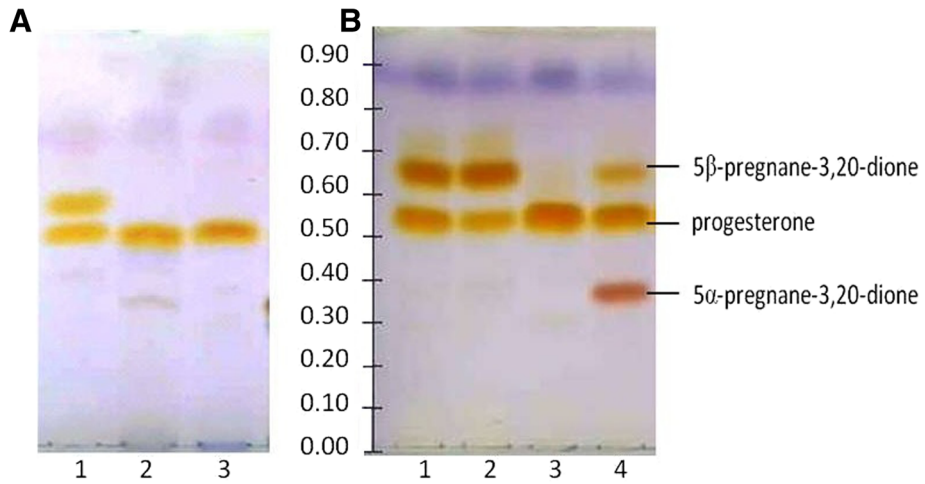

Fig. 5 Analysis of different P5ßR enzyme activities by TLC following a 12-h reaction time. **a** 5β-Pregnane-3,20-dione band ($R_f = 0.65$) obtained from progesterone ($R_f = 0.53$) by the recombinant protein of *P. sitchensis* (*lane 1*) compared to negative controls containing heat-inactivated spruce enzyme plus progesterone (*lane 2*) as well as a progesterone sample without enzyme (*lane 3*). **b** Identification of the reaction product, 5β-pregnane-3,20-dione ($R_f = 0.65$), from progesterone ($R_f = 0.53$) after testing recombinant protein of *A. thaliana* (*lane 1*), *Erysimum crepidifolium* (*lane 2*), and *P. sitchensis* (*lane 3*: heat-inactivated enzyme sample; *lane 4*: reference 5α-pregnane-3,20-dione, $R_f = 0.40$, was added to the active enzymatic reaction as an internal control)

from angiosperms but do not prefer the same substrates, suggesting that they play a role in different metabolic pathways. Favouring small substrates, the spruce enzymes could reduce lipid derivatives (e.g. acrolein or methyl vinyl ketone) containing α,ß-unsaturated carbonyl groups. Such volatile substances of limited size were found to trigger the activation of a pathogenesis-related gene and genes involved in metabolism (Almeras et al. 2003). Our current enzymatic exploration (Table 2) indicates that the recombinant P5ßR of *P. sitchensis* reduces methyl vinyl ketone *in vitro* similar to a number of other enzymes from angiosperms.

Conifers also release a broad range of oleoresin terpenes during diverse defence reactions against herbivores and pathogens (for detailed information, see Zerbe and Bohlmann 2014). Amongst them, single molecules offer activated C=C bonds next to carbonyl oxygen, which are likely applicable to an enzymatic reduction by

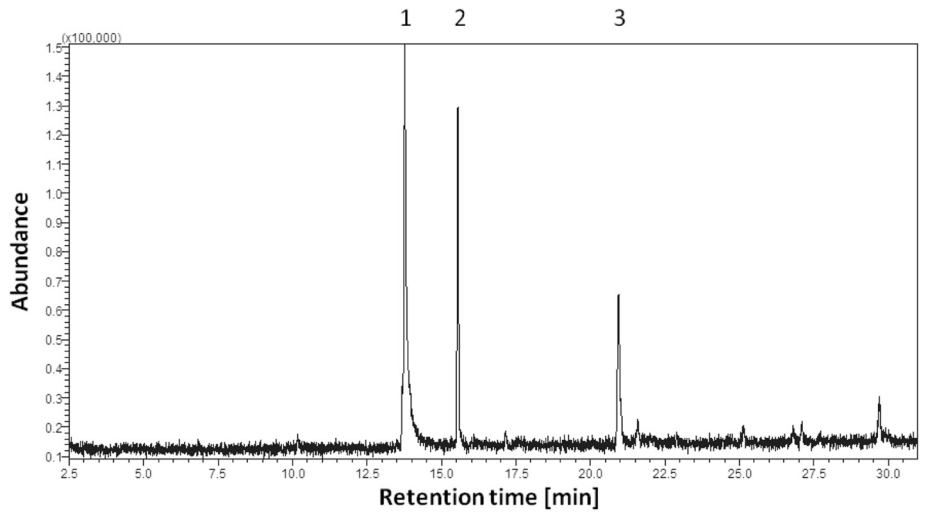

Fig. 6 GC-MS analysis of the enzymatic product obtained with recombinant P5ßR of *P. sitchensis* after a 12-h reaction time. The total ion chromatogram was generated from a sample aliquot previously studied by TLC (Fig. 5b, *lane 4*) and indicates 5ß-pregnane-3,20-dione (*3*; $R_t = 21.1$ min) originating from progesterone (*2*; $R_t = 15.5$ min). The 5α-pregnane-3,20-dione (*1*; $R_t = 13.8$ min) was added to the active reaction as an internal control. It is not produced by enzymatic catalysis as shown in Fig. 5. Peak identification was possible because the substances were tested alone (not shown). Minor peaks do not represent pregnane-like compounds on the basis of a MS data comparison

Table 2 A compilation of individual parameters of recombinant P5ßR enzymes from *Picea sitchensis* (r*Ps*P5ßR), *Arabidopsis thaliana* (r*At*P5ßR), *Erysimum crepidifolium* (r*Ec*P5ßR), and *Medicago truncatula* (r*Mt*P5ßR) with progesterone, 2-cyclohexen-1-one, and methyl vinyl ketone, respectively

Enzyme	Substrate	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} M^{-1})	Preference
r*Ps*P5βR	Progesterone	111	0.59 ± 0.02	0.0053	88.9
	2-Cyclohexen-1-one	88	41.6 ± 3.05	0.4727	
	Methyl vinyl ketone	75	6.0 ± 0.25	0.0800	
r*At*P5βR[a]	Progesterone	268	10.11 ± 2.99	0.0373	15.5
	2-Cyclohexen-1-one	116	66.85 ± 3.78	0.5775	
	Methyl vinyl ketone	n.d.	n.d.	n.d.	
r*Ec*P5βR[b]	Progesterone	77	0.60	0.0077	36.4
	2-Cyclohexen-1-one	518	147	0.2837	
	Methyl vinyl ketone	344	43.8	0.1273	
r*Mt*P5βR[c]	Progesterone	144	30.0	0.2083	1.1
	2-Cyclohexen-1-one	82	19.2	0.2317	
	Methyl vinyl ketone	136	32.4	0.2382	

Substrate preference was calculated dividing k_{cat}/K_M 2-cyclohexen-1-one by k_{cat}/K_M progesterone. The labelled values were taken from the indicated references. The results represent mean values based on three replications done
n.d. not determined
[a]Bauer et al. 2012
[b]Munkert et al. 2011
[c]Munkert et al. 2015

P5ßR activity. Geu-Flores et al. (2012) reported that P5ßR activity is expressed in *C. roseus* during the biosynthesis of a bicyclic monoterpene, namely the iridoid compound nepetalactol (Geu-Flores et al. 2012. Subsequently, it has been demonstrated that the precursor substance 8-oxogeranial is converted in a NADPH-dependent manner by a number of recombinant P5ßR enzymes. This reaction was up to 1.300 times faster than the conversion of progesterone (Munkert et al. 2015). The authors therefore concluded that iridoid synthase activity is an intrinsic feature of all angiosperm P5ßR enzymes that evolved early during evolution. However, structural features currently distinguish iridoid synthase from its close homolog progesterone 5β-reductase (Kries et al. 2015, Alagna et al. 2015). The acceptance of 8-oxogeranial is not restricted to the angiosperms. We found a reaction using the recombinant spruce enzyme as well, which is not yet quantified due to our limited resources. Regarding the diverse catalytic actions, P5ßR was renamed recently as progesterone 5β-reductase/iridoid synthase (PRISE) by Petersen et al. (2015).

Conclusions

If plant P5ßR diversification was related to stress encountered during the transition of plants to growth on land as claimed by Jun et al. (2002), then the investigation of P5ßR proteins from lower plant species should significantly improve phylogenetic studies. Homologous genes from pteridophytes and bryophytes or genes from conifer species will then serve as outgroups to rebuild rooted phylogenetic trees inferring ancestral genes and derived genes, as well as the enzymatic reactions.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors read and approved the final manuscript.

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