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Steroid 5β-reductase from leaves of *Vitis vinifera*: molecular cloning, expression and modelling.

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ABSTRACT: A steroid 5β-reductase gene corresponding to the hypothetical protein of LOC100247199 from leaves of *Vitis vinifera* (var. 'Chardonnay') was cloned and overexpressed in *E. coli*. The recombinant protein showed 5β-reductase activity when progesterone was used as a substrate. The reaction was stereo-selective, producing only 5β-products such as 5β-pregnane-3,20-dione. Other small substrates (terpenoids and enones) were also accepted as substrates, indicating the highly promiscuous character of the enzyme class. Our results show that the steroid 5β-reductase gene, encoding an orthologous enzyme described as a key enzyme in cardenolide biosynthesis, is also expressed in leaves of the cardenolide-free plant *Vitis vinifera*. We emphasize the fact that, on some occasions, different reductases (*e.g.*, progesterone 5β-reductase and monoterpenoid reductase) can also use molecules that are similar to the final products as a substrate. Therefore, *in planta*, the different reductases may contribute to the immense number of diverse small natural products finally leading to the flavor of wine.

KEYWORDS: *Vitis vinifera,* enone 5β-reductase, secondary metabolites, biosynthesis, gene evolution

INTRODUCTION

Previous experiments analyzing the biosynthesis of cardenolides led to the identification of progesterone 5β-reductases (P5βRs; Figure 1) from cardenolide-producing plants.[1-3] The search for orthologous biosynthetic enzymes in non-cardenolide plants was successfully described for the model plant *Arabidopsis thaliana* and later for other closely related species, *e.g.,* from the order Gentianales.[4-7] Functional proteins were found for a number of plant species including cardenolide-free and cardenolide-producing plants, indicating that this class of enzymes may be ubiquitously distributed in the plant kingdom.[6] The hypothesis that orthologous genes may be found in more distinct plant genera of Angiosperms has yet to be proven. The genome of the important crop plant *Vitis vinifera* from rosids, order Vitales, was released recently, and we used this database for a screen (http://www.genoscope.cns.fr/cgi-bin/ggb/vitis/12X/). From a systematic point of view, the order Vitales is far away from Gentianales within the Angiosperms, and *V. vinifera* plants do not produce cardenolides.

Today, more than 200 distinct plant *P5βRs* genes can be found in the NCBI database. They are annotated or of unknown function and all belong to the short chain dehydrogenase/reductase (SDR) family.[8] The SDR enzymes cover a large family of proteins with conserved subdomains or motifs.[9,10] Thorn et al. (2008)[11] defined a novel class of plant SDRs with a different set of sequence motifs and conserved residues (EC 1.3.99.6). *In silico* work and the functional analysis of the role of the subdomains resulted in eight motifs (I to VIII) described by Perez-Bermudez et al. (2010).[12] Motifs I to III are involved in co-substrate binding, discrimination and interaction with NADPH. Motif IV is responsible for stabilizing the β-sheet. Motifs V and VI contain the catalytic tyrosine and lysine residues. Finally, motifs VII and VIII are involved in the proline packs facing the nicotine amide ring of NADPH and for the electrostatic force driving the reaction, respectively.

An increasing number of new entries (including paralogs from a single species) come from genome sequencing projects worldwide. Likely originating from an α-proteobacterium the *P5βR* genes were propagated laterally through nets of ecological interactions.[13] Recombinant enzymes are capable of reducing the active C=C bond of a large variety of substrates by a 1,4-addition.[4,5,7,14] Furthermore, as they also accept small enone-like substrates, it is still under discussion what other reactions may be catalyzed by this class of enzymes.[15,16] The biological function of the enzyme in *Vitis* is not yet established. It may be involved in certain biosynthetic pathways for natural products as small cyclic terpenoid substrates are accepted by other P5βRs, including the iridoid synthase.[17] Additionally, P5βR was described as a function-associated molecular marker at the genus and family levels.[18]

Here, we cloned steroid 5β-reductase genes from leaves of *V. vinifera* (Accession Numbers (Acc.No.) JF460012; KR259636). The overexpressed protein showed a stereo-specific reduction of progesterone to 5β-pregnane-3,20-dione and reduced also small enone substrates. The protein was modelled and was compared to previously overexpressed P5βR proteins. The P5βR gene was used for *Agrobacterium*-mediated transformation and was analyzed for *in planta* expression by RT PCR. The role of the enzyme in *V. vinifera* metabolism is discussed.

MATERIALS AND METHODS

Plant material. *V. vinifera* plants (var. 'Chardonnay' and 'Cabarnet Sauvignon') were grown in the experimental field at FEM-IASMA (San Michele all'Adige, Italy). Embryogenic callus cultivar IT-24/01/12 'Chardonnay' 27 was chosen for *Agrobacterium*-mediated transformation with an antisense binary vector construct. *In vitro* stock cultures were cultivated on GS1CA medium as described by Franks et al. (1998).[19]

RNA extraction and cDNA synthesis. Leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA extraction was carried out using the RNA Spectrum Kit (Sigma GmbH, Taufkirchen, Germany). The synthesis of cDNA was performed employing the RevertAid Kit (Fermentas GmbH, St. Leon-Rot, Germany) for reverse transcription PCR (RT-PCR).

RT-PCR. Standard *Taq* RT-PCR was performed with primers derived from the *EcP5βR* sequence (Acc.No. GU354236) and later created from genomic sequences. A product of 1176 bp in length was generated from the cultivar 'Chardonnay' (KR259636.1) and 1172 bp in length from the cultivar 'Cabarnet Sauvignon' (JF460012.1). A proof reading PCR reaction using the Platinum High Fidelity mix (Invitrogen GmbH, Karlsruhe, Germany) was performed, and the product was directly cloned into the pCR8/Gateway/Topo entry vector. The Gateway® LR Clonase TM enzyme mix kit (Invitrogen) was used to perform the LR recombination reaction for protein over-expression plasmids (pDEST17) or binary vector constructs using the pK7WGIGW2(I) RNAi vector to obtain a hairpin construct. Sequencing was performed at the in-house sequencing platform at FEM-IASMA or by Eurofins GmbH (Ebersberg, Germany). Positive pK7WGIGW2(I)/P5βR plasmids, screened with primers corresponding to the 35S promoter region, were transferred into *Agrobacterium tumefaciens* EHA105 by electroporation (Biorad, Munich, Germany).

Protein extraction. All steps of the crude plant protein preparation were carried out at 4 °C in a cold lab. Leaves of *V. vinifera* were ground to a fine powder using N_2. The powdered plant material was homogenized in extraction buffer (25 mM Tris, 2 mM EDTA, 4 mM DTT, and 10 mM β-mercaptoethanol) for 30 min. The homogenates were squeezed through gauze and were centrifuged at 10.000 x g for 30 min (4 °C). The protein concentration of the supernatant

was determined and was adjusted to 2 mg/mL prior to assaying for P5βR activity. Protein concentration was quantified according to Bradford (1976).[20]

The plasmid pDEST17/P5βR was transformed into the *E. coli* host strain BL21 for over-expression. Cultivation, induction of the bacterial cultures and expression were performed according to Herl et al. (2006).[1] The protein termed r*Vv*P5βR was isolated in its native form according to the manufacturer´s manual (QIAexpressionist™, Hilden, Germany). After purification of the recombinant proteins, the elution buffer was exchanged for the reaction buffer containing 100 mM HEPES-KOH (pH 8.0), 250 mM sucrose, 2 mM EDTA and 10 mM β-mercaptoethanol using PD-10 columns (GE Healthcare, Munich, Germany).

Proteins were analyzed by SDS-PAGE as reported earlier.[21,22] Semi-dry Western blotting was performed according to the QIAexpress Detection and Assay Handbooks (QIAgen, Hilden, Germany) with slight modifications as described.[23] Development and detection took place via the C-digit (LI-COR, Lincoln, USA) scanner. The membrane was incubated for 5 min at room temperature in a "Working Solution" of equal parts of peroxide solution and luminol solution according to the manufacturer's manual. Thereafter, the membrane was placed upside down on the scanner with a scan time of 12 min at high intensity. The generated image was evaluated via Image studio software (LI-COR).

Progesterone 5β-reductase assay. The enzyme assay was conducted as described by Herl et al. (2006).[1] In a final volume of 1000 µL, the standard assay contained: 945 µL protein fraction (0.2 mg/mL recombinant protein, 2 mg/mL plant crude extract), 6.4 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 32.1 mM glucose-6-phosphate, 42 nkat glucose-6-phosphate dehydrogenase and 0.3 mM progesterone. Heat-inactivated samples (10 min, 100 °C) served as controls. Samples were pre-incubated for 10 min at 40 °C before progesterone was added. The reaction was terminated with 1 mL dichloromethane after 2 h at 40 °C. The organic phase was collected and evaporated at room temperature, and the residue

was dissolved in 50 µL ethanol (96 %) or 100 µL acetone for TLC or GC-MS analysis, respectively.

Chromatographic analysis. The above described ethanol concentrates were spotted on TLC plates (silica gel 60), and plates were developed with dichloromethane: ethyl acetate (9:1) for the analysis of pregnanes. Pregnane spots were visualized with anise aldehyde reagent as described earlier.[24]

GC-MS samples were analyzed on a GC Hewlett-Packard HP 6890 MSD Type 5890A using helium as the carrier gas (flow rate 1 mL/min) and an OPTIMA®5 column (30 m x 0.25 mm x 0.25 µm). The temperature program started with an initial 4 min at 200 °C, ran with 20 °C/min up to 290 °C, and finished with an additional increase of 4 °C/min up to 300 °C. The auto sampler injection volume was 1 µL. Pregnanes were detected in full-scan mode by a mass detector and identified comparing their retention times (Rt) and fragmentation patterns with those of authentic compounds. The samples were analyzed in SIM mode, where specific mass fragments were analyzed. Quantification was carried out based on an internal standard (21-hydroxypregnenolone). The program for iridoid compounds started with 60 °C and ran with 5 °C/min up to 150 °C, 20 °C/min gradient up to 240 °C, 20 °C/min up to 290 °C and 5 min isothermal at 290 °C.

***Agrobacterium tumefaciens* strains.** *A. tumefaciens* EHA105 was used for all experiments. Bacteria were grown on LB medium supplemented with the appropriate antibiotics.[25] For all bacterial cultures, 50 mg/L rifampicin was used; in addition, 50 mg/L spectinomycin was used to select for the pK7WGIGW2(I) binary vector for RNAi expression. *Agrobacterium* was cultured in a rotary shaker maintained at 28 °C.

157 **Callus transformation.** Transformation of 'Chardonnay' was performed following the
158 procedure described by Dalla Costa et al. (2014).[26] *Agrobacterium* cultures containing binary
159 vectors were grown in shaking culture for 24 h at 28 °C. Cells were pelleted at room
160 temperature, followed by re-suspension in induction media with 100 μM acetosyringone to an
161 adjusted OD_{600} of approximately 0.5 - 0.7. After 3 h of further incubation at 28 °C, the
162 medium was replaced by centrifugation and resuspension of the *Agrobacterium* cells in liquid
163 GS1CA medium.[19] Embryogenic tissue was inoculated with the suspension of *Agrobacterium*
164 for 7 min at 28 °C. Co-cultured embryogenic callus was blotted on sterile tissue paper and
165 transferred to GSC1A charcoal plates for 48 h co-cultivation at 22 °C in the dark. The co-
166 cultured tissues were washed 4-5 times with liquid culture medium supplemented with
167 Timentin® in 1 mg/mL and transferred in GS1CA plates. After the co-cultivation period, the
168 tissue was transferred to a selection medium containing 0.5 mg/mL Timentin® and the
169 selection antibiotic. Every 3 weeks, the embryogenic tissue was transferred to fresh medium.
170 Regenerated shoots were excised from the callus and transferred to selection medium (SM)
171 with 10 μM 6-benzylaminopurine, 1.5 % sucrose and 1.5 % bacto agar. The cultures were
172 maintained under 94.5 μmol m^{-2} s^{-1} cool white light and 16 h light photoperiod at 22 °C. As
173 soon as roots became visible, the plantlets were placed in MAGENTA® under normal light
174 conditions on SM basal medium for further growth.
175
176 **Analysis of transgenic plants.** For molecular analysis, plantlets grown on SM were selected,
177 and genomic DNA was isolated. The leaves frozen in liquid nitrogen were ground to a fine
178 powder using a pre-chilled mortar and pestle. DNA extraction was carried out using the GE
179 Healthcare DNA Kit (GE Healthcare, Munich, Germany). Presence of the transgene was
180 confirmed by PCR using primers depicted in Table 1.
181

qPCR. *VvP5βR* gene expression of transformed and control plants was performed using real-time PCR. The gene-specific primers were designed based on the *VvP5βR* sequence (Acc.No. JF460012.1; Table 1). qPCR DNA amplification and analysis were carried out using the Bio-Rad CFX96 System with Bio-Rad CFX Manager software version 3.0 (Bio-Rad Laboratories, Hercules, CA). The SSoFast Master Mix (Bio-Rad) was used, and a total reaction volume of 12.5 µL, including 6.25 µL of Master Mix (Bio-Rad), 0.4 µM of each primer, 3.25 µL of water and 2 µL of cDNA, was applied in all reactions following the manufacturer's method. qPCR conditions were 5 min at 98 °C, followed by 40 cycles of 5 s at 98 °C, 5 s at 58 °C, 5 s at 60 °C and 10 s at 76 °C, and followed by 98 °C for 30 s and a melting curve detection with an increment of 0.2 °C from 65 °C to 95 °C. The qPCR efficiency of each gene was obtained by analyzing the standard curve of a cDNA serial dilution of that gene. To normalize the expression data, two *V. vinifera* reference genes, namely actin (*ACT*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were used.[27] Transcript levels of *VvP5βR* were calculated with the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

RESULTS AND DISCUSSION

P5βRs were thought to play a key role in cardenolide biosynthesis (Figure 1).[28,29] This assumption was based on the observation that an enzyme isolated from *Digitalis purpurea* (Plantaginaceae) leaves was capable of reducing progesterone stereo-specifically to 5β-pregnane-3,20-dione (Figure 1).[30] However, Lindemann and Luckner[31] demonstrated that P5βR is also active in pro-embryogenic masses of *Digitalis lanata* and *Digitalis* cell cultures not capable of producing cardenolides.[32] This finding encouraged us to check the occurrence of P5βR in a cardenolide-free plant genus, such as *Vitis* that, from a systematic point of view, is far from *Digitalis*. Furthermore, *Vitis* plants contain a large number of secondary metabolites that represent potential substrates for P5βR reactions, *e.g.* geranial, and small enones may be substrates as shown earlier.[15-17,33] Lindner et al.[34] demonstrated the conversion of native substrates by both the iridoid synthase and the P5βR enzymes, suggesting a cyclization via a Michael addition mechanism. This reaction may open the way for the synthesis of a number of secondary products also typical for wine flavor. However, *V. vinifera* contains secondary metabolites such as stilbenes, proanthocyanidins, anthocyanins, flavonols and others.[35]

A putative homolog of *Digitalis* P5βR was annotated in the recently published draft genome of *Vitis* with 72 % sequence identity (http://www.genoscope.cns.fr/cgi-bin/ggb/vitis/12X/gbrowse/vitis). *Vv*P5βR is located on chromosome 18. A minimum of two *P5βR* genes reside in the genome of Brassicaceae (*e.g.*, *A. thaliana*, *E. crepidifolium,* and *Draba azoides*), and in other plant families up to 7 functional P5βRs have been described (*e.g.*, *Medicago sativa*, *Catharanthus roseus*), representing at least a small gene family.[18,23]

A nucleotide sequence alignment of this *Vv*P5βR with other known 5β-reductase genes showed that all of the eight regions characteristic for this gene family are present in the *Vitis* gene (Figure S1/2).[12] The full length *Vv*P5βR gene from the cultivar ‘Chardonnay’ and the

cDNA of 'Cabarnet Sauvignon' covering the full open reading frame of 1172 bp were cloned. In comparison to the chromosome 18 sequence, an intron of 94 bp is located between 7891434 bp to 7891527 bp on the negative strand in the latter. Therefore, we focused on the *VvP5R1* clone from 'Chardonnay' (KR259636; Figure S2). Nucleotide sequence homology was found to a number of functionally characterized steroid reductases from other plant species. Approximately 70 to 73 % homology was observed to P5βRs of the plant genera from asterids, *e.g.*, *Hoya carnosa* (73 %, GU354231.1), *Gomphocarpus fruticosus* (73 %, GU354238.1), *Asclepias curassavica* (72 %, GU354230.1) and *Calotropis procera* (73 %, GU479996.1). The homology is slightly higher for P5βRs from more closely related genera from rosids, *e.g.*, *Corchorus olitorius* (75 %; HM192827.1) and *Brassica oleracea* (77 %, JQ608337; for comparison see also Munkert et al.[18]).

The *VvP5R1* gene was cloned into the expression vector pDEST17 for expression in *E. coli* strain BL21. The overexpressed P5βR has a calculated theoretical molecular weight of 47.74 kDa. A corresponding band could be assigned to just below the 49 kDa marker band after induction with IPTG on an SDS PAGE gel (Figure 2). The recombinant *Vv*P5βR was detected by Western blot using an anti-mouse IgG-peroxidase antibody (Figure 2B). The catalytic activity was demonstrated by TLC (Figure 3) and was quantified with GC-MS (Figure 4). The enzymatic activity for the substrate progesterone was 5.8 ± 2.8 µkat/kg protein for the recombinant enzyme (kcat: 0.005 s^{-1}), and the activity in *V. vinifera* leaves was calculated to be approximately 3.3 ± 2.8 µkat/kg protein (kcat: 0.003 s^{-1}). Enzymatic parameters for r*Vv*P5βR were compared to functionally related plant enzymes, *e.g.*, r*Dc*P5βR (*Digitalis canariensis*) with 50.1 ± 3.2 µkat/kg (Acc.No. DQ218315, kcat: 0.037 s^{-1}) and r*At*P5βR (*Arabidopsis thaliana*) with 620 ± 6.4 µkat/kg for (Acc.No. EF579963, kcat: 0.457 s^{-1}).[5] Slow enzymatic activities were reported also for other sterol enzyme (*e.g.*, C24-methyltransferase) from soybean with a kcat of 0.01 per sec.[36]

248 Temperature and pH dependencies of the recombinant *Vv*P5βR were determined using
249 progesterone as a substrate. The optimal temperature was at 40 °C to 45 °C. The pH optimum
250 was 8.0 with half-maximum activities at approximately pH 7 and 8.5. This matches with our
251 previously reported results obtained with the recombinant forms of *Dl*P5βR (pH 7.8/40°C),
252 r*At*P5βR (pH 8.0/45 °C) and r*Ec*P5βR (pH 8.0 – 8.5/45 °C).[1,4,7]
253 Terpenoid structures and their metabolic variability were described for *V. vinifera*.[37,38]
254 The *Vv*P5βR has the potential to reduce small enones stereo-specifically as shown for a
255 number of related enzymes in this class.[5,7,15,16] Interestingly, the core structure of the substrate
256 occurs in a number of potential substrates that are responsible for the flavor of wine.[33] Recent
257 publications also point to the fact that this class of enzymes is on one hand a promiscuous
258 enzyme and on the other hand capable of functioning in more than one specific pathway
259 contributing to the enormous number of metabolic products in plants.[39] Geu-Flores et al. [17]
260 showed that iridoid structures (*e.g.*, 8-oxogeranial) have been reduced by cyclases in a
261 reaction that parallels the 1.4-reduction of progesterone by P5βR. Several plant P5βR
262 enzymes catalyze the reaction of 8-oxogeranial *in vitro*. Therefore, *in planta*, the different
263 reductases may contribute to the immense number of diverse small natural products (SNPs)
264 and the flavor of wine (Figure 5). Two molecules were tested under standard conditions as
265 potential substrates. Citral, which is a mixture of the stereo-isomers geranial (citral A) and
266 neral (citral B), was reduced to citronellal (Figure 6). *β*-Ionone, which is formed by oxidative
267 cleavage of carotenoids and belongs to the C_{13}-norisoprenoids, was not accepted as a substrate
268 in our assays with the recombinant protein.
269 Many authors reported that the aroma profiles of grapevine contain hundreds of
270 different aromatic compounds, among them substrates with the core structure.[40-42] The
271 majority of the characterized enzymes catalyzed the synthesis of several final products.[43] We
272 emphasize the fact that, on some occasions, different reductases (*e.g.*, P5βR and
273 monoterpenoid reductase) can also use molecules that are similar to the final product as a

substrate. Sweetman et al. [44] analyzed the 4,185 transcripts from *V. vinifera* and found a high number of differentially expressed genes from secondary metabolic pathways that were upregulated during grape development including genes that are involved in the formation of modified amino acids, aromatic compounds and phenylpropanoids/stilbenes. For example, for the resveratrol synthesis alone, 12 phenylalanine ammonia lyases (PALs), three cinnamic acid 4-hydroxylases (C4Hs), 12 4-coumarate:CoA ligases (4CLs) and 38 stilbene synthases (STSs) were found, indicating a high number of related enzymes catalyzing similar reactions. The biosynthesis of iridoids is another example demonstrating that P5βR accepts a number of small terpenoids.[16,17] Generally, the enzymatic reduction of substrates (like progesterone and/or 2-cyclohexen-1-one) is a key reaction typical for nearly all biosynthetic pathways (*e.g.*, menthol biosynthesis).[45,46]

In addition, the amino acid alignment of plant enone 5β-reductase resulted in a high sequence identity (up to 96 %). Moreover, motifs described for SDR,[9,14] *Dl*5βPR[11] and others[5] suggested the existence of well-defined, highly conserved motifs with defined functions. Perez-Bermudez et al.[12] published eight diagnostic motifs for *D. purpurea*. However, no functional proof for the newly suggested motifs was reported. Therefore, the functional *Vv*P5βR was also modelled using the crystal structure of *D. lanata* (PDB ID: 2V6G) as a template (Figure 7). The two models were highly congruent, and the substructure of the motifs fit perfectly for both models. Single amino acid changes were visible but do not concern functional sequence motifs.[6,23]

Bauer et al. [6] demonstrated the activity-guided improvement of the catalytic efficiency of *Digitalis lanata* P5βR (r*Dl*P5βR) and proposed experiments to convert the weak r*Dl*P5βR into a strong r*At*P5βR-type enzyme.[5] The results revealed at least three hot spot amino acids (Tyr156, Asn205 and Ser248) within a conserved signature of 17 amino acids, that do have a direct influence on the catalytic activity.[6] In this respect, *Vv*P5βR differs in two of the three amino acid positions (Phe156 for Tyr and Ile205 for Asn), and its activity was predicted to be

low. Indeed, the catalytic activity was shown to be much lower than the highly active *A. thaliana* recombinant P5βR (r*At*P5βR).

Expression of r*VvP5βR* was demonstrated by semi-quantitative reverse transcription PCR. The gene transcript was detectable in leaves, stems and roots (Figure 8). To elucidate the physiological role of P5βR, we transformed embryogenic calli (cultivar IT-24/01/12 'Chardonnay' 27) with an RNAi vector construct. Plants were available for analysis after a regeneration period (approximately 17 months). One regenerated line named GT13/5 showed significantly reduced *Vv5βPR* transcript levels (Figure 9). In addition, leaves from the regenerated *Vitis* plant were visually screened for an altered vein pattern. Indeed, Jun et al.[47] described the involvement of the *VEP1* gene in vascular strand development in *A. thaliana* and showed a reduced complexity in the venation pattern of the cotyledons and leaves, which was mainly due to the reduced number of the minor veins and their incomplete connection. The total number of branching points (NBPs) in a leaf can represent the complexity of leaf venation.[48] Striking differences between wild type and RNAi plants could not be detected. However, a reduction of the intensity of the vascular network (Figure 9) was observed. The tendency of vein patterning intensity indicated an involvement of *Vv*P5βR in the formation of leaf veins analogous to *Arabidopsis VEP1*. Experiments and analysis are still in progress. The regenerated transgenic lines will now enable targeted and untargeted metabolome analysis with the aim to investigate the physiological role of *Vv*P5βR and understand the involvement of the enzyme in the different relevant pathways.

As far as the role of P5βR in cardenolide metabolism is concerned, convincing evidence has not yet been supplied. We propose that P5βR is not a key enzyme in cardenolide metabolism as suggested from *in silico* work, but may be involved in other pathways as well.[11,12] The unambiguous demonstration of *P5βR* gene expression and the catalytic activity in cardenolide-free *V. vinifera* (and other plants) supports this assumption.[5] Further

experiments using *V. vinifera* metabolome analysis will be conducted to show the influence of knockout and/or knockdown of the *P5βR* gene on its specific metabolic pattern.

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Notes

The authors declare no competing financial interest.

Supporting Information Statement

Fig. S1. 'Cabarnet Sauvignon' P5βR (JF460012) vs. 'Chardonnay' P5βR (KR259636) sequence alignment. **Fig. S2**. Amino acids sequence alignment of P5βRs of lane 1 - *Vitis vinifera* derived from Acc.No. JF460012 and lane 2 - *Digitalis lanata* derived from Acc.No. AY585867. Conserved motifs I to VIII in bold are indicated according to Perez-Bermudez et al. (2010). This material is available free of charge via the Internet at http://pibs.acs.org.

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Figure captions

Fig. 1. Reaction catalysed by progesterone 5β-reductase (P5βR).

Fig. 2. A - Expression of recombinant *Vv*P5βR in *E. coli* on SDS-PAGE (12 %); Coomassie stained: Lane M, molecular weight marker; Lane 1, bacterial homogenate not induced by IPTG; Lane 2, bacterial homogenate induced by IPTG (r*Vv*P5*β*R1 arrowed); **B -** Western blot of r*Vv*P5βR: Lane 1, r*Vv*P5βR, Lane M, molecular blotting marker.

Fig. 3. TLC analysis of the enzymatic P5βR activity assays (2 hours). Lane 1- progesterone reference, R_f = 0.53; Lane 2- active assay of r*Vv*P5βR; Lane 3- control after treatment at 100 °C for 10 min.; Lane 4 - reference of the final product 5β-pregnane-3,20-dione, R_f = 0.6.

Fig. 4. A: GC-MS analysis of an r*Vv*P5βRs enzyme assay (2 h, 40 °C). (1) - 5β-pregnane-3,20-dione Rt = 9.929 min; (2) - progesterone Rt = 10.637 min. Only 5β-pregnane-3,20-dione but not its 5*α*-derivative is formed enzymatically. (3) – IS, internal standard 21-hydroxypregnenolone Rt = 11.399. **B**: GC-MS analysis of a heat inactivated r*Vv*P5βR enzyme assay with progesterone as substrate (2 h, 40 °C). (1) - Progesterone Rt = 10.620 min. (2) – IS, internal standard 21-hydroxypregnenolone Rt = 11.369.

Fig. 5. Structures of potential substrates and products as important chemical compounds that are responsible for varietal aroma in wine. Both monoterpenes and C_{13}-norisoprenoids are formed from the common precursor mevalonate. The catalytic core structure necessary for the P5βR reaction is shown in red.

Fig. 6. A: GC-MS analysis of the r*Vv*P5βR enzyme assay with citral as substrate (2 h, 40 °C). Compound 1 - citral B/neral (Rt = 11.47 min); compound 2 - citral A/geranial (Rt = 12.249); compound 3 - product citronellal (Rt = 20.353). **B**: GC-MS analysis of a heat inactivated r*Vv*P5βR enzyme assay with citral as substrate (2 h, 40 °C). Compound 1- citral B/neral (Rt = 11.47 min); compound 2- citral A/geranial (Rt = 12.249).

Fig. 7. Atomic model of *Vv*P5βR based on the PDB ID 2V2G (EF579963).[11] Substrate and co-substrate (progesterone, NADPH in blue) have been docked into the binding pocket.

Fig. 8. Expression pattern of the *Vv*P5βR gene *in planta*. RT-PCR analysis of *Vv*P5βR expression in R (roots), S (stems) and L (leaves). Actin gene used as a loading control. RT-PCR performed with template RNA from *V. vinifera* 'Chardonnay' plants.

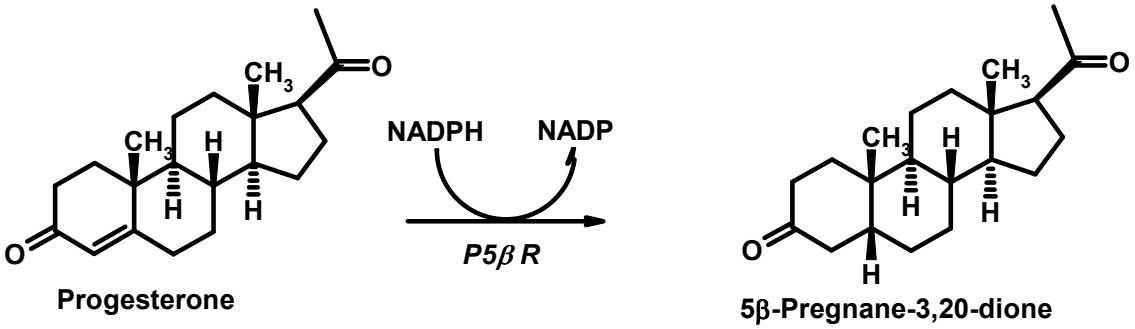
Fig. 9. Expression of *Vv*P5βR in regenerated plantlets. Transcript levels of *Vv*P5βR in 'Chardonnay' wild type (WT) and RNAi plants GT13/5 were determined by qPCR and normalized using the expression of the reference genes *GAPDH* and *ACT*. Bars indicate mean expression levels of three replicates ±SD. * indicates p-value < 0.05. Leaves vein pattern in WT and GT13/5. The leaves were cleared for visualisation of the vein pattern. The RNAi plant GT13/5 showed in comparison to the WT reduction of vein intensity.

Table 1. Primers used for the amplification of *V. vinifera* P5βR and for the amplification of the 35S promoter region and qPCR.

Name	Nucleotide sequence	Tm
*Vv*P5βRdir	ATGAGTTGGTGGTGGGCTGGAG	53 °C
*Vv*P5βRrev	TCAGGGAGGAATGAGTTTGTGAC	50 °C
Promdir	CGCACAATCCCACTATCCTT	60 °C
Promrev	ACACGTGAGCGAAACCCTAT	58 °C
qVvP1213dir	CGATCATCCCGTCGAGTACA	58 °C
qVvP1312rev	GACGTAGAAGACGTGGGTAACGT	58 °C
qGAPDHdir	TTCTCGTTGAGGGCTATTCCA	53 °C
qGAPDHrev	CCACAGACTTCATCGGTGACA	54 °C
qVvACTdir	CTTGCATCCCTCAGCACCTT	54 °C
qVvACTrev	TCCTGTGGACAATGGATGGA	52 °C

Figures

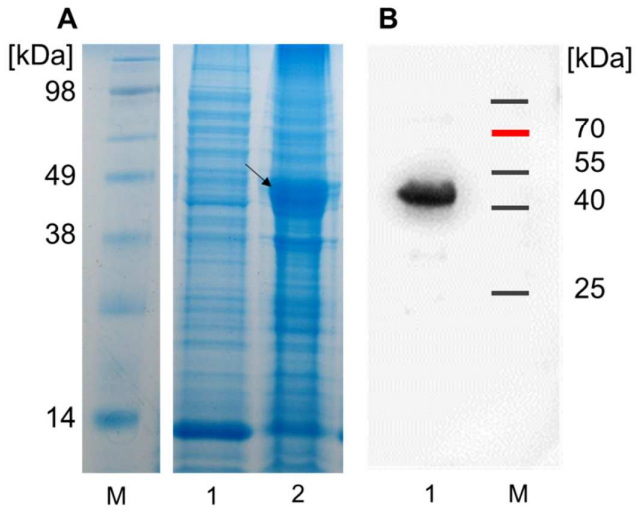
Fig. 1.



Progesterone

5β-Pregnane-3,20-dione

Fig. 2.



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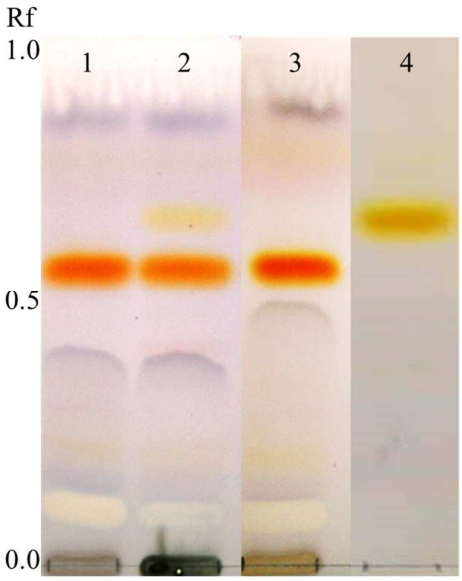
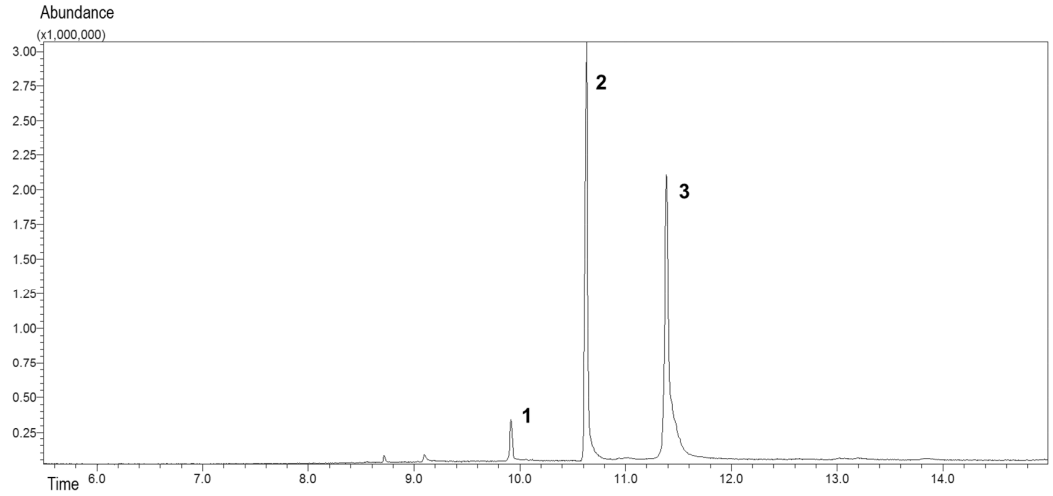


Fig. 4.

A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2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
1
2
3
Time
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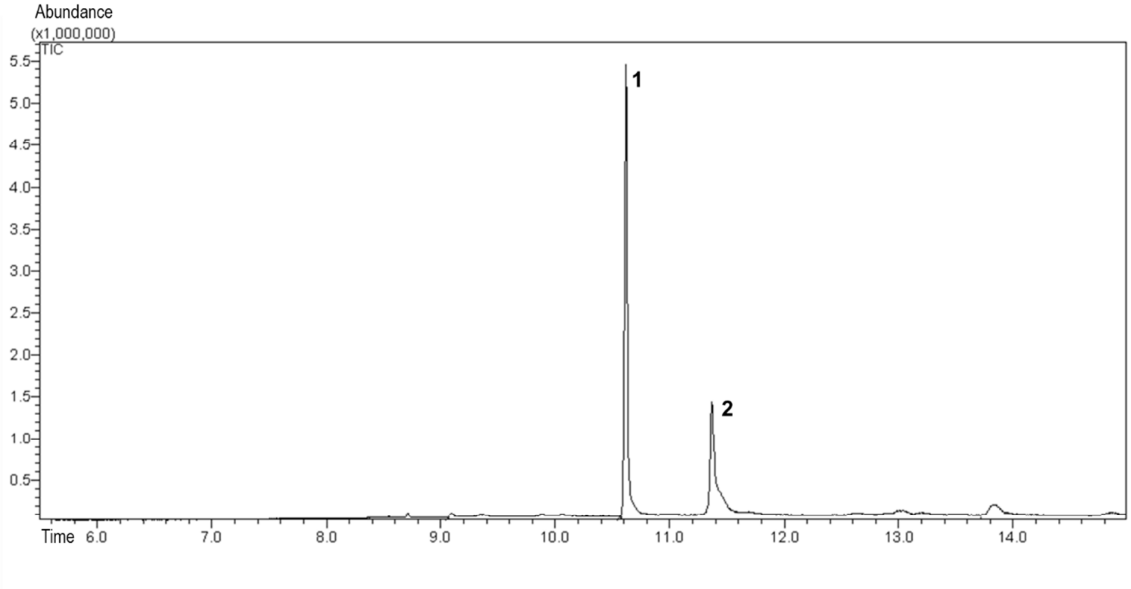
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0

B


Abundance
(x1,000,000)
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5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1
2
Time
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7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0

Fig. 5.

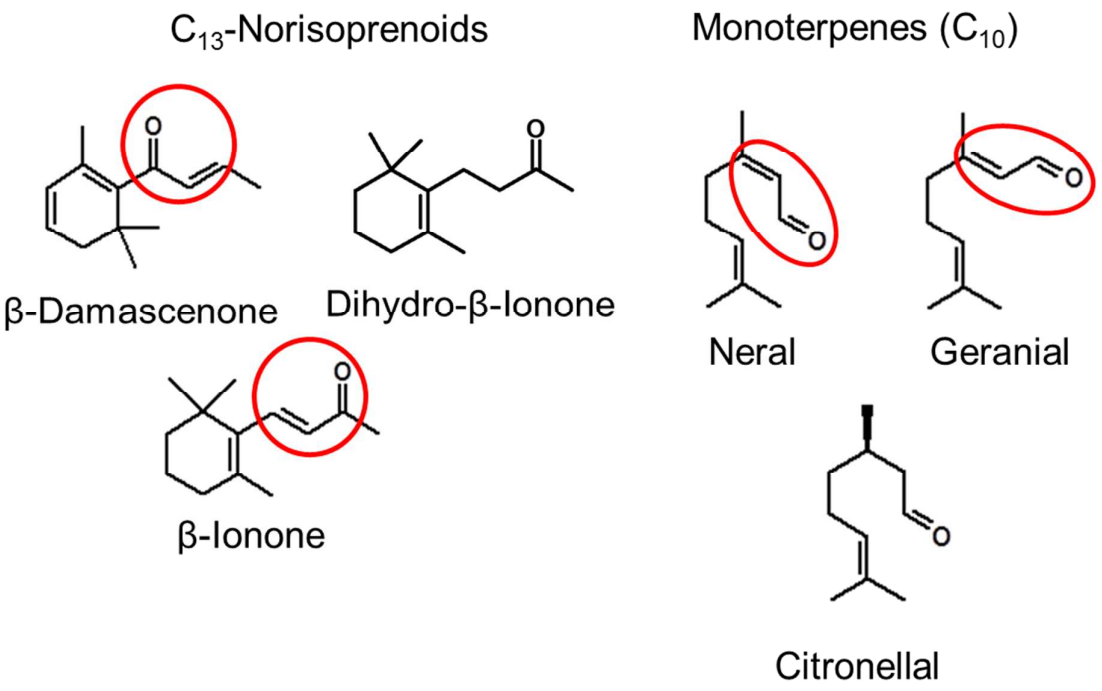
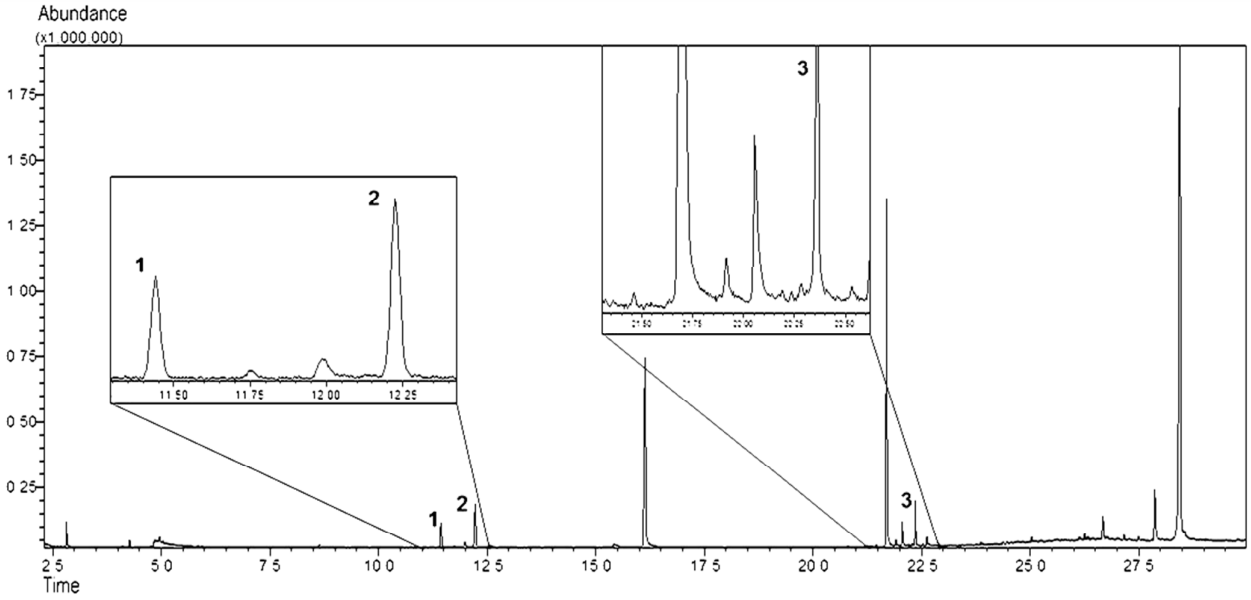

C13-Norisoprenoids
Monoterpenes (C10)
β-Damascenone
Dihydro-β-Ionone
β-Ionone
Neral
Geranial
Citronellal

Fig. 6.

A


Abundance
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11.50
11.75
12.00
12.25
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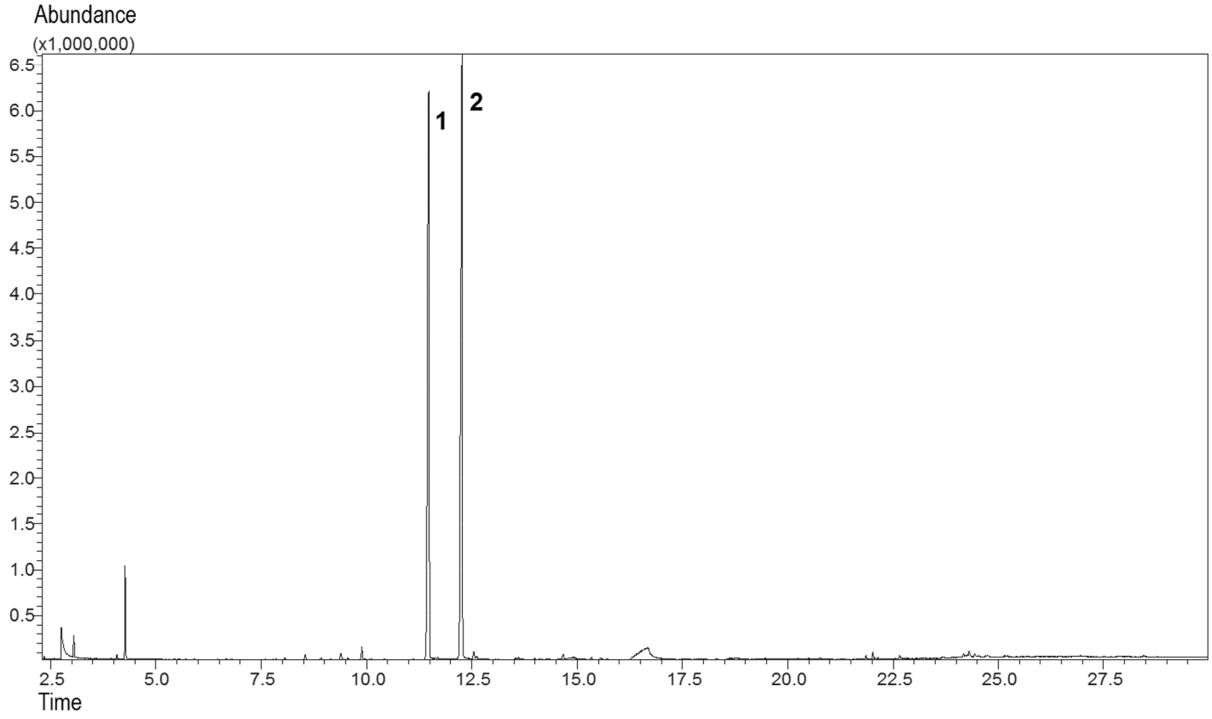
15.0
17.5
20.0
22.5
25.0
27.5
Time

B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0.5
1
2
2.5
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15.0
17.5
20.0
22.5
25.0
27.5
Time

Fig. 7.

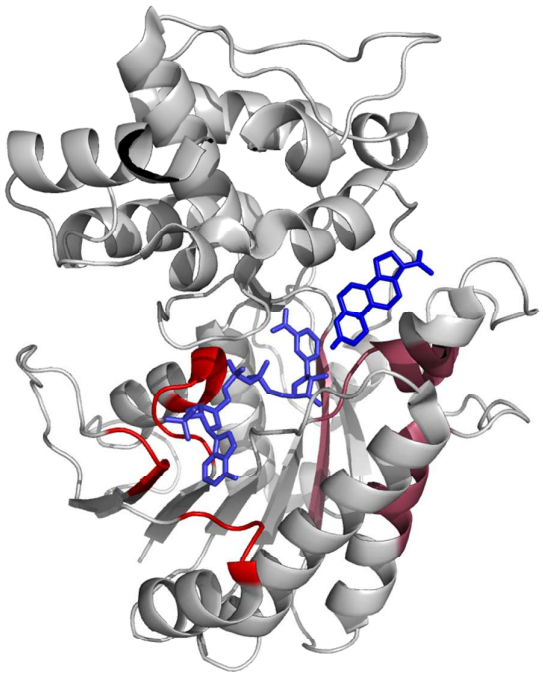
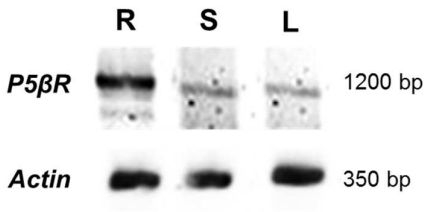


Fig. 8.



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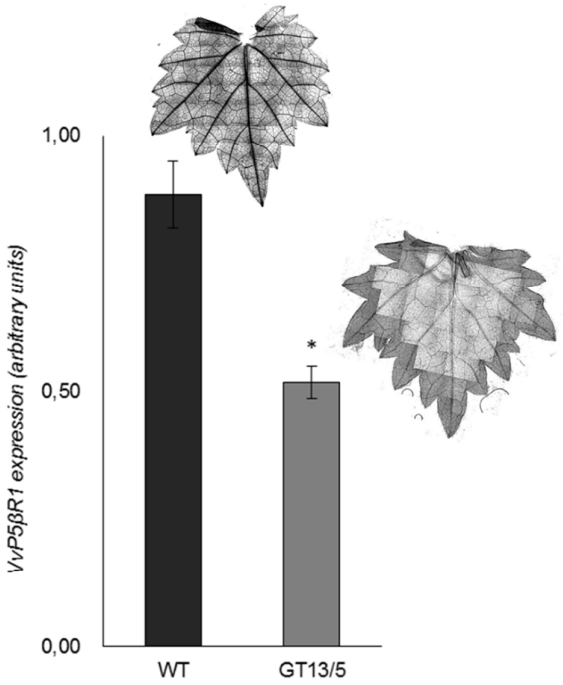

TOC Graphic