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Epoxidation, hydroxylation and aromatization is catalyzed by a peroxygenase from *Solanum lycopersicum*

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ABSTRACT

Plant peroxygenase (PXG) oxidizes unsaturated fatty acids by transferring an oxygen atom of a hydroperoxide to the double bond, thereby providing epoxides. In this work we investigated the potential of a PXG from tomato (*Solanum lycopersicum*, SlPXG) to catalyze the oxidation of a variety of natural products. A *SlPXG* gene was cloned from tomato, heterologously expressed in yeast and the membrane bound recombinant SlPXG protein was used as enzyme source. Unsaturated fatty acids, fatty acid derivatives, and terpenes were epoxidized by SlPXG in the presence of various hydroperoxides exclusively at their *cis*-double bonds. Terpenes with *p*-menthene skeleton were transformed in different ways depending on their molecular structures. R-(+)- and S-(−)-limonene were converted to R-(+)-limonene-*trans*-1,2-epoxide (97%) and *cis*-S-(−)-limonene-1,2-epoxide (88%), respectively whereas α-terpinene was hydroxylated to *cis*-1,4-dihydroxy-*p*-menth-2-ene and γ-terpinene was aromatized to *p*-cymene. In the last reaction the hydroperoxide served as hydrogen acceptor rather than an oxygen donor. PXG appears to be a versatile biocatalyst able to perform different kinds of oxidation reactions. As no cofactors like NAD(P)H are required and H_2O_2 is an environmentally friendly oxidant, PXG enables new applications for the synthesis of fine chemicals from renewable resources.

1. Introduction

Plant oils and fatty acids are appreciated educts for industrial applications [1,2]. Besides oil and carbohydrate, which are compounds of the plant's primary metabolism, the plant secondary metabolites are an important resource for the industry, too. Terpenes belong to the largest and most diverse group of plant secondary compounds. The main sources for terpenes are the essential oils of coniferous, citrus fruits and terebinth [1,2]. They are used as aroma, fragrances, natural insecticides, solvents and pharmaceutical active compounds [1]. Terpenes and other natural products can be used themselves or they can be chemically and/or biotechnologically converted to new molecules. Especially the oxidation of natural products is an important reaction which can be done chemically by heavy metal catalysts or biotechnologically by cell cultures or enzymes [3–5].

Enzymatic oxidations are generally catalyzed by members of the enzyme class 1 (oxidoreductases). Peroxygenases are one subclass of oxidoreductases and are widespread in the fungal and plant kingdom. Peroxygenases from fungi (EC 1.11.2.1) are able to catalyze hydroxylations of aromatic compounds and fatty acids and were investigated due to their promising biocatalytic applications. Numerous articles about these unspecific/aromatic peroxygenases can be found in the literature dealing with physiological, catalytic, phylogenetic and molecular aspects of the enzymes (EC 1.11.2.1) including hydroxylation and epoxidation of terpenes, alkenes and diverse other substrates [6–8]. However, plant peroxygenases (PXG) belong to group EC 1.11.2.3 of the oxidoreductases. They were first discovered in isolated microsomes from pea, *Pisum sativum*, and oxidize substrates by using hydroperoxides as oxidant [9].

The catalytically active proteins from fungi and plants contain a heme molecule in the active site, which is involved in the heterolytic cleavage of the hydroperoxide O—O bond to form a ferryl-oxo complex [10–12]. While the hydroperoxide is reduced to an alcohol, the oxygen atom of the ferryl-oxo complex is transferred to substrates such as aromatic nitrogen compounds, organic sulfur compounds or C=C double bonds of unsaturated fatty acids. Consequently, this oxygen transfer leads to hydroxylations, sulfoxidations or epoxidations, respectively [11–14]. Plant PXG is a membrane protein of the endoplasmic reticulum (ER) and oil bodies and binds calcium in a helices E and F (EF)-hand motive, characteristic for caleosin proteins [11]. In the plant metabolism PXG is part of the oxylipin pathway. After the action of a lipoxygenase, which catalyses the dioxygenation of fatty acids to fatty acid hydroperoxides, PXG subsequently uses these fatty acid hydroperoxides for

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epoxidation reactions [11,13,14]. Plant PXG is able to transfer one oxygen atom to the C=C double bond of unsaturated fatty acids (intermolecular transfer) or to the C=C double bond of the fatty acid hydroperoxide (intramolecular transfer). After epoxidation, epoxide hydrolases can cleave the formed epoxide to yield a diol. The pathway delivers to a mixture of oxidized fatty acids called oxylipins. These molecules are monomers for cutin biosynthesis and phytoalexins which are involved in the defence of the plant against biotic and abiotic stresses [15–17].

Recently, plant PXG protein was isolated from oat seeds (*Avena sativa*) to investigate the epoxidation of a variety of fatty acids and alkenes for biotechnological purposes [18,19]. The present work was undertaken to test the application of a recombinant PXG from tomato (*Solanum lycopersicum*) for the biotechnological oxidation of natural lipids like fatty acids and terpenes. A PXG gene was cloned from *S. lycopersicum* and the recombinant protein was expressed in *Saccharomyces cerevisiae* to provide a reliable enzyme source. A qualitative and quantitative substrate screening was performed and products were identified by GC–MS, LC–MS and NMR. SlPXG converted a number of lipid compounds and catalyzed different kinds of oxidation reactions.

2. Experimental

2.1. Chemicals

All chemicals were used as received from Fluka, Merck, Sigma–Aldrich and Roth.

2.2. Cloning and expression of recombinant PXG from tomato

The cloning and transformation of the *SlPXG* gene and cultivation of *Saccharomyces cerevisiae* INVSc1 cells (Invitrogen) for the heterologous protein expression were performed as described [20].

2.3. Protein crude extract of yeast

The cell pellet from 100 ml culture was washed with 10 ml breaking buffer (50 mmol/l sodium phosphate buffer, pH 7.4, 1 mmol/l EDTA, 5% glycerol, 1 mmol/l phenylmethylsulphonylfluoride, PMSF). After centrifugation (1500 × *g* and 4 °C) for 5 min the pellet was re-suspended in 4 ml breaking buffer containing 0.01% Tween® 20. An equal volume of glass beads (0.25–0.5 mm, Roth, Karlsruhe, Germany) was added and the suspension was vortexed for 30 s, followed by 30 s on ice, which was repeated eight times. The suspension was centrifuged at 2000 × *g* and 4 °C for 5 min. The pellet was washed twice with 4 ml breaking buffer containing 0.01% Tween® 20 and the supernatants were combined.

2.4. Isolation of yeast microsomes

The isolation of the yeast microsomes was done as described [21] except for the re-suspension buffer which consisted of 100 mmol/l Tris, pH 7.5 and 1 mmol/l PMSF. The different protein fractions were subjected to SDS-PAGE followed by Coomassie staining. Enzyme assays were performed with oleic acid **1a** and H_2O_2 to determine the fractions showing the highest activity. Protein concentration was determined [22].

2.5. Screening of fatty acids as substrates for SlPXG

Microsomal protein containing SlPXG (50 μg) was incubated with 2 mmol/l fatty acid (1 μmol in 10 μl methanol) and 2.5 mmol/l H_2O_2 (1.25 μmol) in 500 μl sodium acetate buffer (10 mmol/l, pH 6, 2% glycerol) for 20 min at 40 °C. Three controls were performed with microsomal proteins from empty vector yeast cells, without proteins and without H_2O_2, respectively. The products were extracted twice with each 500 μl CH_2Cl_2, the organic layer was separated and dried under a stream of nitrogen. For methylation of free carboxyl groups, the pellet was solved in 300 μl MeOH, mixed with 150 ml trimethylsilyldiazomethane (2 mol/l) and incubated for 60 min at room temperature. The samples were dried by Speedvac, re-dissolved in 200 ml *n*-hexane and analyzed by Trace GC Ultra gas chromatograph connected to a Trace DSQ mass spectrometer (2.12). Samples containing oleyl alcohol **3a** were solved in 60 μl MeOH (30%) and analyzed by LC–MS (2.13). The pH optimum was determined by varying the pH values of the reaction in steps of 1 between pH 4 and 6 in sodium acetate buffer (10 mmol/l, 2% glycerol) and between 7 and 9 in Tris buffer (10 mmol/l, 2% glycerol), whereas **1a** and H_2O_2 served as substrates. The temperature optimum was determined by varying the reaction temperature in steps of 10 °C between 0 and 80 °C in sodium acetate buffer (10 mmol/l, pH 6, 2% glycerol). For determination of saturating curves the substrate concentrations were varied between 0.02 and 2 mmol/l in sodium acetate buffer (10 mmol/l, pH 6, 2% glycerol) with 50 μg microsomal protein containing SlPXG. The values of saturating curves were used for apparent K_m value calculation with excel solver (Microsoft).

2.6. Screening of other natural products as substrates for SlPXG

Microsomal protein containing SlPXG (100 μg) was incubated with 1 mmol/l substrate (0.5 μmol in 10 μl methanol) and 2.5 mmol/l H_2O_2 (1.25 μmol) in 500 μl sodium acetate buffer (10 mmol/l, pH 6, 2% glycerol) for 40 min at 40 °C. Three controls were performed with proteins from empty vector yeast cells, without proteins and without H_2O_2, respectively. The products were extracted and analyzed by GC–MS as described for fatty acids, except that the methylation reaction was omitted. The dried pellet was solved in 100 μl MeOH.

2.7. Activity assay for the oxidation of fatty acids

Microsomal protein containing SlPXG (50 μg) was incubated with 0.4 mmol/l fatty acids (0.1 μmol in 10 μl methanol) and 2 mmol/l H_2O_2 (0.5 μmol) in 0.25 ml sodium acetate buffer (10 mmol/l, pH 6, 2% glycerol, 0.01% Tween® 20) for 20 min at 40 °C. A 0.1 ml aliquot of this mixture was added to 1.9 ml NADH solution (final concentration 125 μmol/l; in 100 mmol/l Tris-HCl, pH 8; Sulfite-kit, R-Biopharm AG, Darmstadt, Germany). The OD_{340} was measured and 5 μl of peroxidase solution (Sulfite-kit) was added. After 1 h at room temperature the OD_{340} was measured again. The amount of H_2O_2 was calculated from OD_{340} values as described in the manual of the kit. The activity of SlPXG was determined by comparison of the H_2O_2 levels at the start and end of the SlPXG reaction. Background activity was measured using methanol instead any substrate and substracted from the enzyme activity.

2.8. Activity assay for the oxidation of terpenes and other natural products

Microsomal protein containing SlPXG (50 μg) were incubated with 0.1 mmol/l substrate (0.05 μmol in 10 μl methanol) and 2.5 mmol/l H_2O_2 (1.25 μmol) in 500 μl sodium acetate buffer (10 mmol/l, pH 6, 2% glycerol) for 40 min at 40 °C. Products and remaining substrates were extracted by head space SPME (65 μm polydimethylsiloxane/divinylbenzene, fused silica, SUPELCO Analytical, Bellefonte, USA) for 20 min. Quantification was performed by Trace GC Ultra gas chromatograph connected to a Trace DSQ mass spectrometer (2.12). The conversion of substrate was calculated by a calibration curve created with different substrate

concentrations (0.01, 0.05, 0.075 and 0.1 mmol/l) under the same conditions. The activity of the SlPXG was quantified by the decrease of the substrate level corrected with an empty vector control.

2.9. Identification of unknown oxidation products by NMR

Crude protein extract containing SlPXG (10 mg) was mixed with substrates (13.6 mg α-phellandrene **10a**, 13.6 mg α-terpinene **11a**, or 50 mg α-linolenic acid **6a**) in 100 ml sodium acetate buffer (10 mmol/l, pH 6). The reaction was started by adding 25.5 μl H_2O_2 (9.79 mol/l) and stirred at 40 °C for 5 h. The reaction was monitored by taking samples of 0.5 ml every 30 min, which were extracted with CH_2Cl_2 and measured by GC–MS as described above. Additional amounts of 25.5 μl H_2O_2 (9.79 mol/l) were added every 0.5 h, and 13.6 mg **10a** was added after 1.5 and 3 h. The products of α-terpinene **11a** were extracted three times with 50 ml diethyl ether, whereas the products of α-phellandrene **10a** and α-linolenic acid **6a** were extracted with 50 ml CH_2Cl_2. The organic phases were dried with sodium sulphate and slowly concentrated with the help of a Vigreux column at 35 °C. The products of **6a** were methylated by trimethylsilyldiazomethane (2.5) and the terpene products were directly subjected to silica gel chromatography to purify the oxidation products. A mixture of n-hexane and ethyl acetate (3:2 for terpene products and 8.5:1.5 for products of **6a**) was used. Fractions were monitored by GC–MS. The fractions containing the purified oxidation product were combined, slowly dried at room temperature and the residue solved in $CDCl_3$ with 0.03% tetramethylsilane (TMS). ^1H and ^{13}C NMR analysis was performed using a Bruker DMX-400 spectrometer (Bruker, Rheinstetten, Germany). The spectrometer frequencies were 500 MHz and 125 MHz for the determination of chemical shifts of ^1H and ^{13}C nuclei, respectively. The chemical shifts were determined using TMS as internal standard in the proton dimension and the carbon signal of $CDCl_3$ in the carbon dimension. The assessment of NMR spectral data was done using the software MestReNova (www.mestrelab.com).

2.10. Chemical oxidation

The oxidation of substrates with *m*-chloroperoxybenzoic (*m*CPBA) acid was performed as described [23].

2.11. Oxidation with different hydroperoxides as oxidant

The oxidation of **1a**, S-(−)-limonene **9a**, **11a** and γ-terpinene **12a** was done as described (2.5; 2.6), but instead of H_2O_2 0.75 mmol/l *tert* butyl hydroperoxide or 0.5 mmol/l cumene hydroperoxide was used as oxidant. Incubation period, extraction and measurement procedure were identical. For saturating curves, 50 μg microsomal protein containing SlPXG was mixed with 0.5 mmol/l **1a** (0.25 μmol in 10 μl methanol) in 500 μl sodium acetate buffer (10 mmol/l, pH 6, 2% glycerol). The concentration of H_2O_2, *tert* butyl hydroperoxide and cumene hydroperoxide varied from 0.01 to 5 mmol/l, 0.1 to 5 mmol/l and 0.005 to 2 mmol/l, respectively. The mixture was incubated for 40 min at 40 °C and treated as described above for fatty acids. The amounts of the oxidation products were quantified by GC–MS and the values used for apparent K_m value calculation with excel solver.

2.12. GC–MS

Fatty acids and terpenes were analyzed with a Finnigan Trace GC Ultra gas chromatograph (Thermoelectric Corporation, Dreieich, Germany) connected with a FinniganTrace DSQ (Thermoelectric Corporation). A VF-5MS capillary column (5% diphenyl/95% dimethylsiloxane; 30 m; film thickness 0.25 μm; Agilent Technologies, Santa Clara, USA) was used. The carrier gas was helium with a flow rate of 1.1 ml/min. The temperature was maintained at 100 °C for 5 min, then raised to 280 °C at a rate of 5 °C/min, and finally held at 280 °C for 10 min. The mass spectrometer parameters were as follows: electron energy 70 eV; emission current 100 μA; ion source temperature 250 °C. The mass spectra were measured in the mass range from *m*/*z* 50 to 600. Limonene oxide enantiomers were resolved with a gaschromatograph 450-GC (Agilent Technologies, Santa Clara, USA) connected to a FID. A chiral capillary column (octakis-(2,3-di-*O*-butyryl-6-*O*-*tert*-butyldimethylsilyl)-γ-cyclodextrin solved in SE 52, 30 m) was used. The carrier gas was hydrogen with a flow rate of 1 ml/min. The column temperature was maintained at 40 °C for 5 min, then raised to 210 °C at a rate of 1.5 °C/min, and finally held at 210 °C for 5 min. The temperature of the FID was 280 °C; the combustion gas consisted of synthetic air (300 ml/min) and hydrogen (30 ml/min). The molecular weights of unknown terpene oxides were determined with a Hewlett Packard GC HP 5890 Series II (GMI, Ramsey, USA) connected with sector field mass spectrometer MAT 95S (Finnigan MAT, Bremen, Germany) for chemical ionization. A DB-5 capillary column (5% diphenyl/95% dimethylsiloxane; 30 m; film thickness 0.25 μm Agilent Technologies, Santa Clara, USA) was used. The carrier gas was helium with a flow rate of 1 ml/min. The column temperature was maintained at 35 °C for 2 min, then raised to 185 °C at a rate of 5 °C/min, then raised to 250 °C at a rate of 15 °C/min and finally held at 250 °C for 10 min. The mass spectrometer parameters were as follows: electron energy 70 eV; ion source temperature 200 °C. Reactant gas was isobutane.

2.13. LC–MS

An Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and a variable wavelength detector, and connected to a Bruker Daltonics Esquire3000plus ion trap mass spectrometer (Bruker Daltonics, Bremen,Germany) was utilized. A C-18 column (150 mm × 2 mm, particle size 5 μm, Phenomenex, Newport Beach, USA) held at 25 °C was used. The mobile phase was a mixture of water (A) and methanol (B) both containing 0.1% formic acid. The flow rate was 0.2 ml/min. After 5 min at 30% B the gradient went from 30% to 100% B in 45 min. Finally, the gradient decreased to 30% B in 10 min. The detection wavelength was 234 nm. The electrospray ionization voltage of the mass spectrometer ranged from 4000 V to −500 V. Nitrogen was used as dry gas at a temperature of 330 °C and a flow rate of 9 l/min. The full scan mass spectra were measured in the range from *m*/*z* 30 to 800 with a scan resolution of 13,000 *m*/*z*/s. The collision gas for the mass spectrometry was helium with a collision voltage of 1 V. Mass spectra were acquired in both positive and negative ionization modes. Analyses of LC–MS.data were performed using the Bruker Daltonics software.

2.14. Mass spectra and NMR data

9,10-Epoxy octadecanoic acid methyl ester **2b**. RT 14.9 min. MS (EI): *m*/*z* 312 (M^+, 1%), 155 (74), 97 (45), 83 (46), 74 (88), 69 (76), 55 (100).

9,10-Epoxy octadecanol **3b**. RT 43.1 min. MS (APCI): *m*/*z* 307 ($M+Na^+$), MS/MS 307 (100%), 305 (39).

9,10-Epoxy-12-octadecenoic acid **4b** (methylated). RT 11.6 min. MS (EI): *m*/*z* 310 (M^+, 1%), 155 (18), 109 (24), 95 (50), 83 (44), 79 (37), 69 (59), 67 (78), 55 (100).

12,13-Epoxy-9-octadecenoic acid **4c** (methylated). RT 11.5 min. MS (EI): *m*/*z* 310 (M^+, 1%), 164 (15), 136 (19), 121 (24), 95 (58), 81 (90), 79 (45), 67 (84), 55 (100).

12,13-Epoxy-10-octadecenoic acid **5b** (methylated). RT 13.4 min. MS (EI): *m*/*z* 310 (M^+, 1%), 225 (24), 207 (19), 199 (36), 149 (27), 139 (41), 121 (27), 99 (100), 81 (58), 55 (98).

15,16-Epoxy-9,12-octadecadienoic acid **6b** (methylated). RT 11.7 min. MS (EI): *m*/*z* 308 (M^+, 1%), 108 (32), 107 (12), 93 (42), 81 (22), 79 (100), 67 (45), 57 (37), 55 (52); NMR: δ_H (500 MHz; $CDCl_3$; Me_4Si) 1.07 (t, *J* = 7,5, 3H), 1.25–1.41 (m, 8H), 1.5–1.66 (m, 2H), 2.06 (q, *J* = 6.6, 7.1, 2H), 2.2–2.28 (m, 2H), 2.32 (t, *J* = 7,5, 2H), 2.40–2.45 (m, 2H), 2.82 (dd, *J* = 6.9, 7.0, 2H), 2.87–3.01 (m, 2H), 3.68 (s, 3H), 5.31–5.55 (m, 4H); δ_C (125 MHz; $CDCl_3$; Me_4Si) 10.6 (C18), 21.1 (C17), 26.1 (C3), 26.2 (C14), 27.2 (C11), 27.2 (C8), 29 (C4), 29.1 (C7), 29.2 (C5), 29.6 (C6), 34.1 (C2), 51.4 (C19), 56.5 (C16), 58.3 (C15), 124.2 (C12), 127.3 (C10), 130.5 (C9), 130.8 (C13), 174.2 (C1).

11,12-Epoxy eicosanoic acid **7b** (methylated). RT 16.5 min. MS (EI): *m*/*z* 340 (M^+ 0.2%), 199 (10), 183 (20), 167 (18), 155 (29), 149 (41), 87 (52), 81 (54), 74 (76), 69 (72), 55 (100).

R-(+)-limonene-*trans*-1,2-epoxide **8b**. MS (EI): *m*/*z* 152 (M^+, 1%), 137 (5), 108 (44), 94 (68), 79 (47), 67 (61), 43 (100).

S-(−)-limonene-*cis*-1,2-epoxide **9b**. MS (EI): *m*/*z* 152 (M^+, 3%), 137 (30), 119 (15), 109 (40), 93 (42), 81 (34), 79 (41), 67 (77), 43 (100).

1,2-Epoxy-*p*-menth-5-ene **10b**. MS (EI): *m*/*z* 152 (M^+, 3%), 126 (65), 111 (85), 109 (37), 95 (47), 93 (28), 71 (66), 43 (100); NMR: δ_H (500 MHz; $CDCl_3$; Me_4Si) 0.85–0.95 (2d, *J* = 6.8, 6H), 1.34 (s, 3H), 1.62–1.68 (m, 1H), 1.75–1.82 (m, 2H), 2.05–2.1 (m, 1H), 3.45–3.50 (m, 1H), 5.65–5.72 (m, 2H); δ_C (125 MHz; $CDCl_3$; Me_4Si) 19.00 (C9, C10), 25.91 (C3), 30.17 (C7), 31.49 (C8), 42.58 (C4), 69.13 (C1), 73.65 (C2), 132.09 (C6), 134.04 (C5).

5,6-Epoxy-*p*-menth-1-ene **10c**. MS (EI): *m*/*z* 152 (M^+, 3%), 127 (17), 111 (40), 100 (95), 85 (55), 84 (41), 71 (100), 69 (70); NMR: δ_H (500 MHz; $CDCl_3$; Me_4Si) 0.86–1.00 (2d, *J* = 7.0, 6H), 1.55–1.65 (m, 1H), 1.80 (s, 3H), 2.0–2.05 (m, 1H), 2.12–2.16 (m, 2H), 3.46–3.51 (m, 1H), 3.74 (q, *J* = 7.0, 1H), 5.46–5.48 (m, 1H); δ_C (125 MHz; $CDCl_3$; Me_4Si) 16.61 (C9, C10), 18.49 (C7), 26.39 (C3), 31.77 (C8), 48.10 (C4), 69.37 (C5), 71.05 (C6), 128.75 (C2), 139.13 (C1).

Cis-1,4-Dihydroxydihydroxy-*p*-menth-2-ene **11b**. MS (EI): *m*/*z* 168 (M^+, 0.5%), 141 (35), 127 (37), 123 (40), 109 (100), 81 (48), 43 (47); NMR: δ_H (500 MHz; $CDCl_3$; Me_4Si) 0.92–1.00 (2d, *J* = 6.9 6H), 1.36 (s, 3H), 1.56–1.62 (m, 2H), 1.74–1.80 (m, 2H), 1.85–1.95 (m, 1H), 5.65 (dd, *J* = 1.5, 10.0, 1H), 5.75 (dd, *J* = 1.5, 10.0, 1H); δ_C (125 MHz; $CDCl_3$; Me_4Si) 16.37 (C10), 17.55 (C9), 27.09 (C5), 29.69 (C7), 33.50 (C6), 37.44 (C8), 67.25 (C1), 71.58 (C4), 133.46 (C3), 135.46 (C2).

p-Cymene **12b**. MS (EI): *m*/*z* 134 (M^+, 31%), 120 (11), 119 (100), 117 (18), 115 (9), 91 (34), 77 (9), 65 (9).

Nerol-2,3-epoxide **15b**. MS (EI): *m*/*z* 170 (M^+, 0.5%), 121 (17), 109 (72), 95 (26), 93 (31), 82 (31), 69 (100), 67 (56), 41 (85).

3,4-Dihydroxy bisabolol **16b**. MS (EI): *m*/*z* 256 (M^+, 0.5%), 220 (8), 151 (11), 127 (6), 109 (100), 93 (14), 82 (50), 69 (56), 43 (67).

Bisabololoxid B **16c**. MS (EI): *m*/*z* 238 (M^+, 1%), 179 (14), 161 (36), 143 (100), 125 (46), 107 (26), 105 (57), 85 (52), 81 (43), 71 (44).

Cis-jasmone-7,8-epoxide **17b**. MS (EI): *m*/*z* 180 (M^+, 3%), 165 (15), 152 (7), 123 (17), 122 (35), 110 (43), 109 (20), 95 (22), 79 (100), 67 (33), 43 (25), 41 (32).

Cis-stilbene-oxide **18b**. MS (EI): *m*/*z* 196 (M^+, 31%), 195 (41), 178 (22), 167 (100), 165 (35), 152 (24), 105 (42), 90 (52), 89 (69), 77 (28).

3. Results and discussion

3.1. Heterologous expression of SlPXG

A *PXG* gene was cloned from tomato (*S. lycopersicum*) and heterologously expressed in yeast (*S. cerevisiae*) [20]. *S. cerevisiae* was chosen as expression host because PXG is a membrane protein of the endoplasmic reticulum (ER) and can be easily isolated with the yeast microsomes, the relict of the ER after cell lysis [11,20,24]. After protein expression and isolation of microsomes, SDS-PAGE analysis showed a protein band at 28 kDa representing SlPXG (calculated molecular weight: 27.9 kDa) (supplementary online material Figure S1). Enzyme activity assays were performed on different fractions obtained during the preparation of the microsomes. Considerable enzymatic activity was detected in the microsomal fraction which was 1.5 times higher than in the crude protein extract. Furthermore, the activity could be increased up to 2.3 times by adding the detergent Tween® 20 probably due to the extraction of the protein from the membrane.

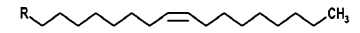
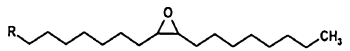
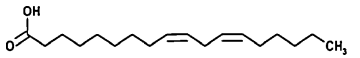
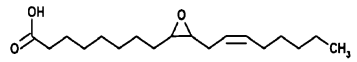
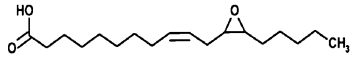
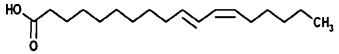
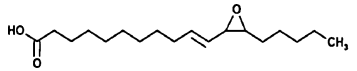
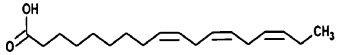
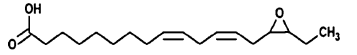
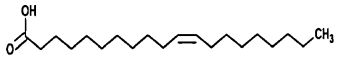
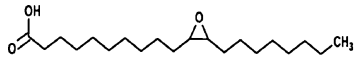
PXG genes from different plant sources have already been expressed in *Escherichia coli*, *Pichia pastoris* and *S. cerevisiae* to analyze their biological functions [11,20,24] and a PXG enzyme has been isolated from oat seed [19] to perform a substrate screening. Similar to the PXG protein sequences from *Arabidopsis thaliana*, *Sesamum indicum*, *A. sativa* and *Oryza sativa* SlPXG contains a transmembrane domain which is localized between arginine 91 and leucine 111 (supplementary online material Figure S2) and explains the membrane integration and the high PXG enzyme activity in the microsomal fraction. Like other PXGs, SlPXG has a heme molecule in the active site and contains conserved histidine residues 69 and 133 which are believed to be involved in heme binding [24] (supplementary online material Figure S2). The heme iron is involved in the oxygen transfer from a hydroperoxide to the substrate. Experiments with the monoxygenase inhibitor tetcyclacis proved the important role of the heme in the catalysis of SlPXG [20].

3.2. Epoxidation of fatty acids and fatty acids derivatives

PXGs are enzymes that oxidize unsaturated fatty acids to *cis*-configurated epoxides [25,26]. Similarly, recombinant SlPXG was able to epoxidize unsaturated fatty acids and their derivatives at their double bonds (Table 1). The pH optimum was determined to be 6 and the temperature optimum 40 °C when using **1a** as substrate. The identity of the products was proven by comparison of the mass spectra with those of reference substances, literature data and chemically (by *m*CPBA) synthesized epoxides. One of the oxidation products of α-linolenic acid **6a** was identified by NMR analysis (Table 1).

1a and **2a** were oxidized by SlPXG to 9,10-epoxy octadecanoic acid **1b** and 9,10-epoxy octadecanoic acid methyl ester **2b**, respectively, as already described for PXGs from *Glycine max* [10] and *Avena sativa* [18,19,24,26]. In contrast to the enzymatic oxidation of oleic acid, where only the *cis*-9,10-epoxy stearic acid was formed, the chemical oxidation by *m*CPBA resulted in *cis*-9,10-epoxy octadecanoic acid (76%) and *trans*-9,10-epoxy octadecanoic acid (24%) (supplementary online material Figure S5). The chemical oxidation of methyl oleate yielded a similar distribution of products. The double bond partly isomerized from *cis* to *trans* configuration during the oxidation by *m*CPBA. Thus, enzymatic epoxidation by SlPXG resulted in purer products. Although the oleyl alcohol **3a** turned out to be a poor substrate and exact quantification of the product yield was not feasible, the product 9,10-epoxy octadecanol **3b** could be clearly detected. Conjugated *trans*-10, *cis*-12-linoleic acid **5a** was only oxidized at the *cis*-double bond to yield 12,13-epoxy-10-octadecenoic acid **5b**, demonstrating the preference of SlPXG for *cis*-double bonds [13,24]. The chemical oxidation of **5a** by *m*CPBA led to the formation of **5b** and a second product, which was putatively identified as the 10,11-epoxide. SlPXG oxidized linoleic acid **4a** to the monoepoxides 9,10-epoxy-12-octadecenoic acid **4b** and 12,13-epoxy-9-octadecenoic acid **4c** in a ratio of 1: 1. The diepoxide was not formed. In contrast, PXG from *G. max* favoured the attack at the 9,10-double bond (72%) [13] whereas PXGs from *A. sativa* and *A. thaliana* produced both monoepoxides and the diepoxide [19,27]. Different regioselectivity of different PXGs was also observed in the case of α-linolenic acid **6a**. This fatty acid was epoxidized by SlPXG to one main and two

Table 1
SlPXG catalyzed transformation of fatty acids and fatty acids derivatives.[a]

Entry	Substrate	Product	Product ratio (%)	Product yield (%)[b]	Activity (nmol/min)[b]	K_m value (mmol/l)[c]	Identification
1							
	R= COOH **1a**	**1b**	100	15	0.74	0.071	Reference
2	R=$COOCH_3$ **2a**	**2b**	100	13	0.66	0.167	Reference
3	R=CH_2OH **3a**	**3b**	100	<1	<0.1	0.05	Synthesis
4	 **4a**	 **4b**	50	9.5	0.46	0.183	[38]
		 **4c**	50	9.5	0.46	0.163	[38]
5	 **5a**	 **5b**	100	23	1.14	0.815	Synthesis
6	 **6a**	 **6b**	68	24	1.18	0.21	NMR
7	 **7a**	 **7b**	100	25	1.24	0.078	Synthesis

[a] Reaction conditions are detailed in Section 2
[b] Determined by consumption of H_2O_2
[c] Apparent K_m values determined by GC–MS except for **3a** which was determined by LC–MS.

side products (68%, 17% and 15%). The main product was purified by silica gel chromatography and identified by NMR as 15,16-epoxy-9,12-octadecadienoic acid **6b** (see 2.14 for NMR data). However, PXG from *A. sativa* [18] and *A. thaliana* [23] mainly formed the 9,10-, 15,16-diepoxide (92%). This indicates that different isoforms of PXGs enable the synthesis of defined epoxides from unsaturated fatty acids. Absolute stereochemistry of epoxy products formed by SlPXG was not determined but PXG4 from *A. thaliana* [27] catalyzed exclusively the formation of (R),(S)-epoxide enantiomers, which is the absolute stereochemistry of the epoxides found *in planta*.

The conversion of fatty acids and the activity of SlPXG were quantified using a coupled enzyme assay. Unsaturated fatty acids were oxidized by SlPXG in the presence of H_2O_2 and the remaining hydroperoxide was reduced by NADH and NADH-peroxidase. The NADH quantity was determined photometrically at 340 nm and used for the calculation of consumed H_2O_2 (Table 1). While SlPXG epoxidized **7a** and **6a** with highest (1.24 and 1.18 nmol/min, respectively) and **1a** and **2a** with lowest activity (0.74 and 0.66 nmol/min, respectively), the substrate preference of PXG from *A. sativa* was in reverse order; highest activity was determined with **1a** and **2a**, while **7a** and **6a** were converted with 75 and 50% relative activity, respectively [24].

3.3. Oxidation of terpenes and volatile natural products

In addition to unsaturated fatty acids also numerous terpenes contain *cis* double bonds and could function as potential substrates for PXGs. Therefore, the enzymatic oxidation of certain terpenes by SlPXG was investigated (Tables 2 and 3).

SlPXG stereoselectively epoxidized R-(+)-limonene **8a** and S-(−)-limonene **9a** to R-(+)-limonene-*trans*-1,2-epoxide **8b** (97% diastereomeric purity) and S-(−)-limonene-*cis*-1,2-epoxide **9b** (88% diastereomeric purity), respectively. The absolute configuration of the products were determined with pure R-(+)-limonene-*trans*-1,2-epoxide **8b** and chemically enriched S-(−)-limonene-*trans*-1,2-epoxide by GC-FID using a chiral capillary column (supplementary online material Figures S3 and S4). Although the conversion of **8a** by the SlPXG is lower (39%) than by heavy metal containing catalysts (75% [28] and 97% [29]) the diastereomeric excess of the SlPXG product is higher (97%) than that in the chemical reaction (max. 89% [28] and 93% [29]). Limonene-1,2-epoxides can be used as fragrances [1,2] or for the synthesis of polycarbonates [30].

Besides limonene, additional terpenes containing a *p*-menthene skeleton were tested as substrates for SlPXG. R,S-α-phellandrene **10a** was epoxidized by SlPXG to at least five products. The molecular mass of every product was determined to be 152 Da by GC-(CI)–MS. Two pairs of products showed the same mass spectra but different retention times which let us suppose that these were diastereomeric forms. Diastereomeric pairs were purified by silica gel chromatography and the NMR spectra of the different products showed (2.14) that SlPXG catalyzed the formation of 1,2-epoxy-*p*-menth-5-ene **10b** (ratio of diastereomers: 26: 13% of total products) and 5,6-epoxy-*p* menth-1-ene **10c** (26: 17%). The fifth product (18% of total products) was not identified but the molecular weight of 152 Da implies that an epoxide or ketone was produced.

Hydroxylation reactions catalyzed by plant PXGs are known for indole, phenol and aniline [9]. This kind of oxidation was also noticed for at least two terpenes (**11a** and **16a**). SlPXG oxidized α-terpinene **11a** to only one product, which was identified as *cis*-1,4-dihydroxy-*p*-menth-2-ene **11b** by NMR and mass spectrometry (2.14). As the endoperoxide ascaridole is known to be rapidly converted into **11b** [31], ascaridole is postulated to be an intermediate of the hydroxylation of **11a** (Fig. 1). The transformation of **11a** to ascaridole has already been demonstrated by an iodide-peroxidase [32] and supports the involvement of ascaridole as intermediate in

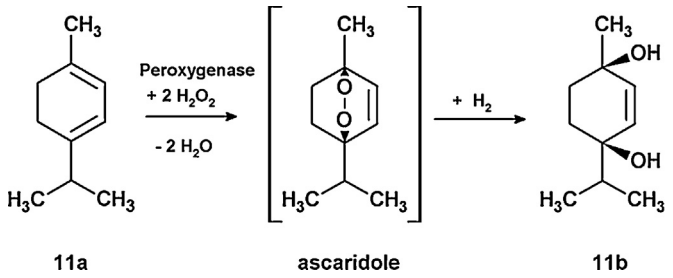


Fig. 1. Hydroxylation of α-terpinene **11a** by SlPXG via the hypothetical intermediate ascaridole.

the hydroxylation of **11a**. The *p*-menthenes γ-terpinene **12a** and α-thujene **14a** were transformed to *p*-cymene (**12b**, **14b**) with 71 and 26% yield, respectively (Tables 2 and 3). SlPXG efficiently catalyzed the dehydrogenation of the cyclic monoterpenoid skeleton of **12a** and **14a** to the aromatic *p*-cymene **12b** rather than an epoxidation or hydroxylation reaction. This is the first report showing that PXGs catalyze an aromatization reaction. The aromatization of **12a** to **12b** was first discovered by incubating **12a** together with leaves of thyme (*Thymus vulgaris*) [33] and is probably catalyzed by cytochrome P450 enzymes in thyme. The *p*-menthenes, which possess a 1,4-cyclohexadiene structure, are well known educts for aromatization reactions. It has been shown that **12a**, 1,4-cyclohexadiene and 1-methyl-1,4-cyclohexadiene react easily with $KMnO_4$ at 0 °C to **12b**, benzene and toluene, respectively [34]. In the oxidation mechanism of 1,4-cyclohexadiene to benzene a cyclohexadienyl cation was proposed to be a transition state [35]. Likewise, in the aromatization reaction catalyzed by SlPXG H_2O_2 seems to act as hydrogen acceptor instead of an oxygen donor, thus enabling the dehydrogenation of the cyclohexadiene ring. Interaction of the heme iron with the single-oxygen donor H_2O_2 can lead to the formation of an iron-oxo intermediate which eventually gets reduced by hydrogen provided by the substrate (Fig. 2). Alternatively, it is conceivable that **12a** is hydroxylated and then may aromatize via water elimination. Compound **12b** is an important solvent and fragrance. Furthermore it can be a platform chemical for other aromatic products [36].

Terpinolene **13a** seemed to be converted to minor amounts of an epoxide or ketone as was deduced from the mass spectrum of the product.

The fact that SlPXG did not oxidize geraniol but nerol **15a** is in accordance to the preference of the enzyme for *cis* double bonds [13]. Nerol-2,3-epoxide **15b** was the only product formed by SlPXG from **15a** (Table 3).

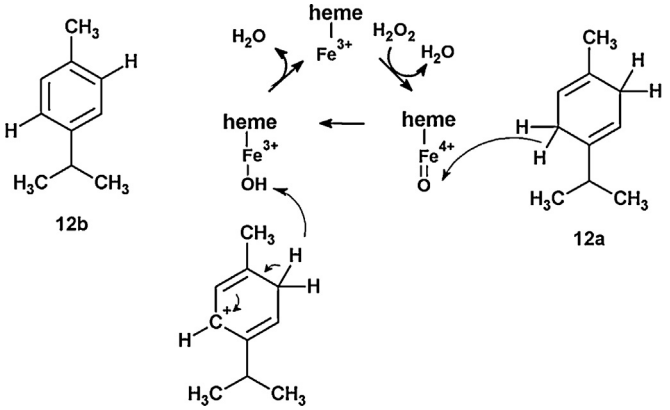
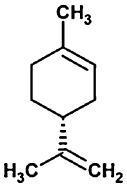
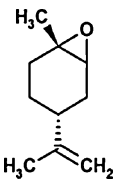
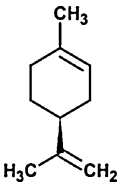
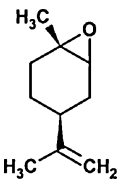
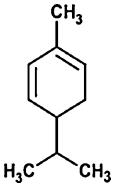
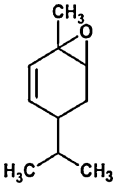
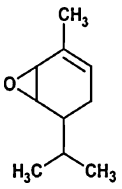
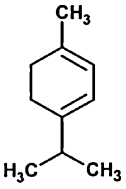
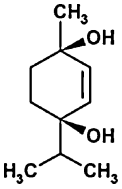
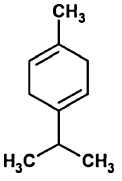
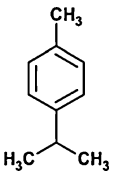
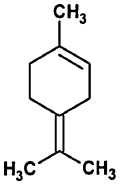


Fig. 2. Postulated reaction mechanism for the aromatization of γ-terpinene **12a** to *p*-cymene **12b** by SlPXG. Only the heme residue of the enzyme is shown for simplicity.

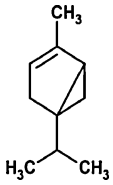
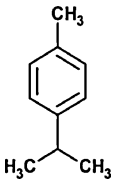
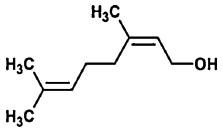
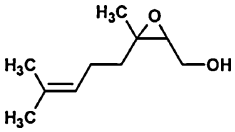
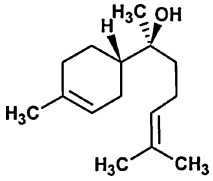
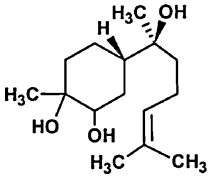
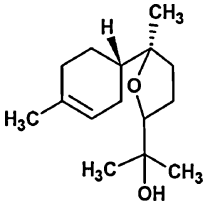
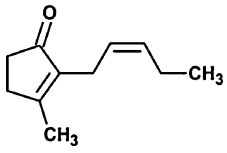
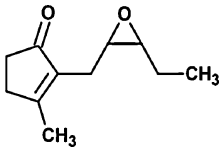
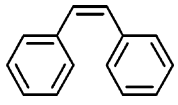
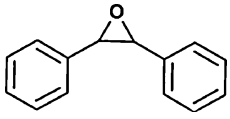
Table 2
SlPXG catalyzed transformation of terpenes and other natural products.[a]

Entry	Substrate		Product		Product ratio (%)	Product yield (%)[b]	Activity (nmol/min)[b]	Identification
8		**8a**		**8b**	97	38	0.48	Reference
9		**9a**		**9b**	88	31	0.39	Reference
10		**10a**		**10b**	39	20	0.25	NMR
				**10c**	43	22	0.28	NMR
11		**11a**		**11b**	100	47	0.59	NMR
12		**12a**		**12b**	100	71	0.88	Reference
13		**13a**	Probably epoxide or ketone		100	7	0.09	

[a] Reaction conditions are detailed in Section 2.
[b] Determined by consumption of substrate.

The sesquiterpene α-bisabolol **16a** was efficiently oxidized by SlPXG (37% residual **16a**). The products are 3,4-dihydroxy-bisabolol **16b** (40%), bisabololoxide B **16c** (35%) and one unidentified compound (25%). The 3,4- and 10,11-epoxide are assumed to be reaction intermediates [37]. Hydrolysis of the 3,4-epoxide yields **16b** whereas the 7-hydroxyl group of the 10,11-epoxide may attack nucleophilically the epoxide at position C10, which results in a 7,10-epoxide and the 11-hydroxyl group of **16c**. In contrast, biotransformation of **16a** by a culture of the fungus *Glomerella cingulate* resulted in the production of numerous products [37].

Table 3
SlPXG catalyzed transformation of terpenes and other natural products[a]

Entry	Substrate		Product		Product ratio (%)	Product yield (%)[b]	Activity (nmol/min)[b]	Identification
14		**14a**		**14b**	50	26	0.33	Reference
15		**15a**		**15b**	100	16	0.20	[28]
16		**16a**		**16b**	40	25	0.32	[37]
				**16c**	35	22	0.28	[39]
17		**17a**		**17b**	100	11	0.14	[40]
18		**18a**		**18b**	100	10	0.12	Reference

[a] Reaction conditions are detailed in Section 2.
[b] Determined by consumption of substrate.

Compound **16c** is a pharmaceutical active ingredient known from German chamomile (*Matricaria chamimilla*). Thus, the synthesis of bisabolol oxides by the SlPXG can open the way for new drugs.

Furthermore, the oxidation of *cis*-jasmone **17a** resulted in *cis*-jasmone-7,8-epoxide **17b**, whereas jasmonic acid and methyl jasmonate were not oxidized by the SlPXG. *Cis*-stilbene **18a** was transformed to *cis*-stilbene oxide **18b**.

The *p*-menthenes α-pinene, 3-carene and S-(+)-carvone were not accepted as substrates by SlPXG despite their *cis* double bonds. Similarly, α-ionone, β-caryophyllene and two aromatic compounds eugenol and isoeugenol were not oxidized by SlPXG.

The calculated SlPXG reactivity ranged from 0.09 to 0.88 nmol/min for the terpene substrates **13a** and **12a**, respectively. Although most of the tested *p*-menthenes and other natural products were epoxidized, it seems that the hydroxylation (entries 11 and 16) and aromatization reactions (entries 12 and 14) proceeded the fastest.

3.4. Comparison of different hydroperoxides

In addition to H_2O_2, cumene hydroperoxide and *tert* butyl hydroperoxide have been described as co-substrates for plant PXGs [9,13,18]. Similarly SlPXG catalyzed the epoxidation of **1a** and **9a**, the hydroxylation of **11a** and the aromatization of **12a** in the presence of the three hydroperoxide co-substrates. The three hydroperoxides produced identical products. The enzymatic reaction was inhibited when the levels of H_2O_2, cumene and *tert* butyl hydroperoxide exceeded 2.5, 0.5, and 0.75 mmol/l, respectively. The apparent K_m value for cumene hydroperoxide was 0.05 mmol/l using **1a** as substrate and corresponded to the value obtained for a PXG from *G. max* [13]. The apparent K_m values for H_2O_2 and *tert*-butyl hydroperoxide were 0.6, and 0.25 mmol/l, respectively. SlPXG showed the highest epoxidation activity of **1a** with H_2O_2 as oxidant (relative product amount 100%), whereas cumene and *tert*-butyl hydroperoxide were less active oxidants (43% and 32%, respectively). Unlike the preference of SlPXG, a PXG from *A. sativa*

showed the highest activity when *tert*-butyl hydroperoxide was used [18]. H_2O_2 is a suitable co-substrate for SlPXG because of its low costs and eco friendliness.

4. Conclusion

A recombinant PXG from *S. lycopersicum* (tomato) is able to oxidize a number of unsaturated *cis*-configurated natural compounds such as fatty acids and terpenes to yield a variety of products. The kind of oxidation reaction catalyzed by PXG depends on the molecular structure of the substrate and includes epoxidation, hydroxylation and aromatization. Thus, PXG provides new opportunities for the biotechnological production of functionalized natural products. The results can find application in whole-cell-production systems and transgenic plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.07.001.

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