Lipids
DOI 10.1007/s11745-013-3815-1

ORIGINAL ARTICLE

A Trichome-Specific Linoleate Lipoxygenase Expressed During Pyrethrin Biosynthesis in Pyrethrum

Aldana M. Ramirez · Ting Yang · Harro J. Bouwmeester · Maarten A. Jongsma

Received: 18 January 2013 / Accepted: 3 July 2013

Abstract The lipid precursor alcohols of pyrethrins—jasmolone, pyrethrolone and cinerolone—have been proposed as sharing parts of the oxylipin pathway with jasmonic acid. This implies that one of the first committed steps of pyrethrin biosynthesis is catalyzed by a lipoxygenase, catalyzing the hydroperoxidation of linolenic acid at position 13. Previously, we showed that the expression and activity of chrysanthemyl diphosphate synthase (TcCDS), the enzyme catalyzing the first committed step in the biosynthesis of the acid moiety of pyrethrins, is trichome-specific and developmentally regulated in flowers. In the present study we characterized the expression pattern of 25 lipoxygenase EST contigs, and subsequently carried out the molecular cloning of two pyrethrum lipoxygenases, *TcLOX1* and *TcLOX2*, that have a similar pattern to *TcCDS*. Only recombinant TcLOX1 catalyzed the peroxidation of the linolenic acid substrate. Just as *TcCDS*, *TcLOX1*, are exclusively expressed in trichomes. Phylogenetic analysis showed that the enzyme shared the highest homology with chloroplast-localized 13-type-lipoxygenases that are involved in maintaining basal levels of jasmonate.

Keywords Pyrethrum · Hydroperoxides · Lipoxygenase · Pyrethrins · Oxylipins · Trichomes · Tanacetum cinerariifolium

Abbreviations

EST	Expressed sequenced tag
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase gene
HAc	Acetic acid
HPLC	High performance liquid chromatography
HPOT	Hydroperoxyoctadecatrienoic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic acid
LOX	Lipoxygenase
PUFA	Poly-unsaturated fatty acid
RACE	Rapid amplification of cDNA ends
RGE	Relative gene expression
RLE	Relative level of expression
RT	Retention time
RTqPCR	Real time quantitative PCR
SDS_PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TcCDS	Chrysanthemyl diphosphate synthase EC 2.5.1.67 from *T. cinerariifolium*
TcGLIP	GDSL-like lipase EC 3.1.1.X from *T. cinerariifolium*
TcLOX	Linoleate lipoxygenase from *T. cinerariifolium*

Introduction

Pyrethrins, the insecticidal constituents of pyrethrum flower extracts, are esters consisting of a combination of

Electronic supplementary material The online version of this article (doi:10.1007/s11745-013-3815-1) contains supplementary material, which is available to authorized users.

A. M. Ramirez · T. Yang · M. A. Jongsma (✉)
Plant Research International, Wageningen University and Research Centre, Wageningen, The Netherlands
e-mail: maarten.jongsma@wur.nl

A. M. Ramirez · T. Yang · H. J. Bouwmeester
Laboratory of Plant Physiology, Wageningen University, Wageningen, The Netherlands

A. M. Ramirez · T. Yang
Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands

Published online: 28 July 2013

either one of two acids (chrysanthemic acid or pyrethric acid), and three alcohols (pyrethrolone, cinerolone, or jasmolone) (Fig. 1). The three alcohols esterified to chrysanthemic acid are collectively known as type I pyrethrins, whereas the esters of pyrethric acid represent type II pyrethrins [1, 2]. The alcohol moieties of pyrethrins resemble the plant hormone jasmonic acid (JA (**4**)), and have been suggested to be derived either from 7-OH-jasmonic acid (**5**) or *Z*-jasmone (**7**) through the oxylipin pathway (Fig. 2) [2]. Specific oxylipins have important regulatory roles in different aspects of plant development [3–5] as well as in plant defense against pathogens and insects [6–8]. The generation of oxylipins is initiated by the action of lipoxygenases/LOX (Linoleate:oxygen oxidoreductase, EC 1.13.11.12), which are non-heme, iron-containing di-oxygenases that catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids (PUFA) containing a (1*Z*, 4*Z*)-pentadiene system, such as linoleic acid, linolenic acid (**1**), or arachidonic acid. Since linoleic and linolenic acid constitute the most common substrates found in plants, LOX are classified with respect to the position (9 or 13) and stereo-specific oxygenation on the hydrocarbon backbone of these fatty acids. Based on the presence of a plastidic transit peptide on their primary structure, LOX can be grouped into two subfamilies [9, 10]. Those enzymes carrying no transit peptide are designated as type-1 LOX, and those harboring putative chloroplast transit peptide sequences are classified as type-2 LOX. All LOX-forms carrying a chloroplast signal peptide (type-2) catalyze the incorporation of oxygen at position 13 (13-LOX) of the fatty acid substrate. Hence, it would be expected from the fact that subsequent steps of the JA biosynthetic pathway are in the plastids that for pyrethrin biosynthesis a type-2, 13-LOX gene is involved.

Considerable progress has been made in the characterization of chrysanthemyl diphosphate synthase (TcCDS), the enzyme catalyzing the first committed step in the biosynthesis of the acid terpene moiety of pyrethrins [11], and GDSL-like lipase TcGLIP, an acyltransferase cloned and demonstrated to be responsible for the esterification of (1*R*,3*R*)-chrysanthemoyl-CoA and (*S*)-pyrethrolone [12]. We recently demonstrated that while the production of the acid moiety of pyrethrins (chrysanthemic acid) by TcCDS and other enzymes occurs in the trichomes, the esterification with the alcohol moiety (pyrethrolone) by TcGLIP happens in the pericarp [13]. However, neither the tissues in which the rethrolone precursors of pyrethrins are produced nor the enzymes catalyzing their first biosynthetic step are known yet.

We also showed that the *TcCDS* and *TcGLIP* genes were highly expressed in the early stages of flower development (stages 2–5), when there was an active accumulation of pyrethrins, and that their expression was down-regulated as the flower matured and the concentration of pyrethrins had reached its maximum (stages 6–7) [13]. Given these observations, we hypothesized that any gene involved in the formation of the alcohol counterpart of pyrethrins would be developmentally regulated in the same way as the *TcCDS* and *TcGLIP* genes. The aim of the present study was to clone pyrethrum LOX enzymes that were regulated in a *TcCDS*-like fashion, and to test the ability of the recombinant enzyme to catalyze the di-oxidation of the linolenic acid substrate.

Materials and Methods

Plant Material and RNA Isolation for EST Library Construction

T. cinerariifolium seeds were originally obtained from Honghe Senju Biology Co. Ltd. (Kunming, Yunnan, China) [14], and propagated without selection at Plant Research International. Plants were grown in the field in a sandy soil, supplied with a regular fertilizer regime, sampling of the flowers took place between the months of June and September.

Ovaries and ovary glandular trichomes were isolated from fresh stage-3 flowers of plant genotype 10. For the isolation of ovaries, ray florets were removed and the

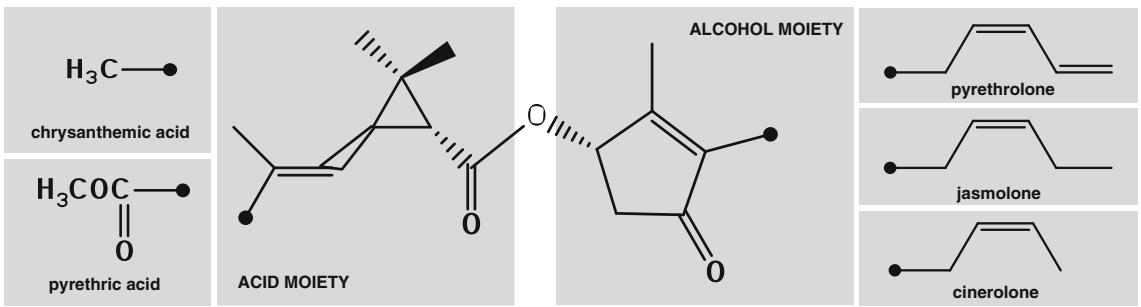

Fig. 1 Chemical structures of natural pyrethrins from *Tanacetum cinerariifolium*. The alcohol moieties, pyrethrolone, cinerolone, and jasmolone, form esters with the acid moieties, chrysanthemic acid and pyrethric acid, yielding the six pyrethrins esters

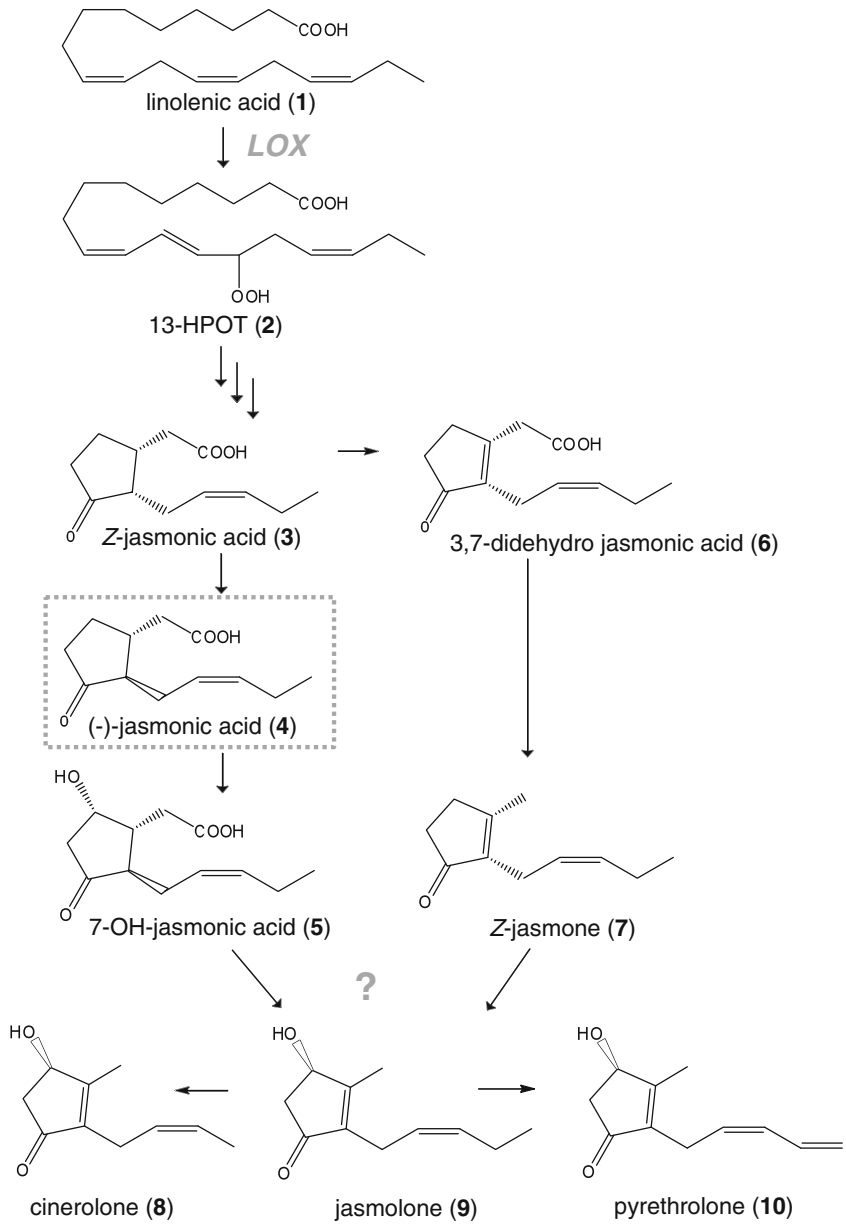

Fig. 2 Proposed biosynthetic pathway of the alcohol moiety of pyrethrins. In *grey* the lipoxygenase (LOX) involved in the first dedicated step leading to cinerolone, jasmolone and pyrethrolone

composite flower heads were cut into half. Corollas of disc florets were excised at the calyx. The remaining ovaries were then isolated by cutting the achenes from the receptacle and immediate collection in a 2-mL Eppendorf tube filled with liquid nitrogen. Trichomes were removed from the excised ovaries with four vortexing pulses of 1 min, intermitted each time by 1 min cooling in liquid nitrogen. Frozen ovaries were spread on a pre-cooled 150-µm mesh mounted over a pre-cooled 50-mL screw-capped tube. The trichomes that passed through the mesh were collected at the bottom of the tube and later used for RNA isolation. Excised ovaries, isolated trichomes and not yet fully developed young leaves were used to isolate RNA. To avoid interference of trichome content, the RNA of each sample was isolated with hexadecyltrimethylammonium bromide (CTAB).

Generation of Three EST Libraries

Three micrograms of total RNA from each tissue was sequenced with 454 sequencing technology by Vertis Biotechnologie AG (Germany) [15]. cDNA libraries of the three different tissues were obtained with random hexamer primers which were labeled with different adapters. The three libraries were normalized, and then sequenced in a single 454 run. This run generated 281,264 reads for the ovary library (60 % of total reads), 87,226 reads for the trichome library (19 % of total reads) and 98,672 reads for

the leaf library (21 % of total reads). After adapter clipping, reads were discarded if the length of the reads was lower than 60 nt. For each library, <3 % of reads were discarded. The remaining reads were clustered and assembled into contigs using CLC Main Workbench (CLCBio, Denmark). In every library, about 40–50 % reads that could not be assembled into contigs were left as singlets. Since all the plant material was harvested from the same plant, the reads from different libraries were pooled together to be assembled. In this way, 458,726 high quality reads from all three libraries were assembled into 27,314 contigs leaving 31.6 % (144,825) reads as singlets. The average length of contigs was 411 bp. Using an in-house bioinformatics facility, potential gene functions of the resulting contigs were identified by blasting against the Nr database of annotated genes, saving the 50 best hits for each contig in a local database which could be interrogated with keywords.

Characterization of *LOX* Candidates by RTqPCR

For RNA extraction, plant tissue was homogenized by adding one pre-cooled grinding ball to each 2-ml Eppendorf tube containing 50–100 mg of frozen plant tissue and using a pre-cooled Mikro-disembrator II (Braun; Germany) for 1 min at maximum speed. After careful removal of the beads, RNA was isolated using TriPure (Roche) and transcribed into cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems) according to the manufacturer's instructions.

RT-qPCR was used to study the expression of 25 candidate *LOX EST* contigs and the chrysanthemyl diphosphate synthase gene (*TcCDS*) in cDNA derived from ovaries at different flower developmental stages and trichomes isolated from ovaries at stage 4 of development. Gene specific primers were designed using Beacon Designer (Palo Alto, CA, USA) with expected product sizes of around 120 bp (Table S1, supplemental data). *T. cinerariifolium* Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) [13] was used for normalization. PCR reactions were prepared in duplicate by mixing in a 500-µL tube, 22.5 µL iQ SYBR green supermix 2x (Bio-rad), 4.5-µL sense primer (3 µM), 4.5-µL antisense primer (3 µM), 11.5 µL deionized water, and 2 µL cDNA template. After vortexing, each mix was distributed into two wells in 20-µL amounts. Quantification of the transcript level was performed in an MyiQ iCycler system (Bio-Rad Laboratories, USA) using a three-step program, which included (1) enzyme-activation at 95 °C for 3 min, (2) 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and (3) 95 °C for 1 min, from 65 to 95 °C for 10 s for dissociation curve analysis. At the end of each run, amplified products were sequenced to verify their identity. Relative gene expression (RGE) values were calculated using the efficiency δCt method [16].

Amplification and Plasmid Construction for Expression in *E. coli*

SMART RACE cDNA amplification kit (Clontech, USA) was used to obtain the 5′ and 3′-region of those six selected EST contigs showing a pattern of expression similar to the pattern of expression of *TcCDS*, according to the manufacturer's instructions. 5′-RACE and 3′-RACE products were cloned into pGEMT-easy vector (Promega, USA) and sequenced. Assembly of the six original fragments and newly sequenced products using SeqMan software resulted in two full-length cDNA sequences, named *TcLOX1* (genbank: KC441523) and *TcLOX2* (genbank: KC441524).

The full-length candidate LOX were amplified from ovary cDNA using high fidelity Phusion polymerase (Finnzymes) and the primers TcLOX1-F, 5′-TTAT**CTGCAG**CATGGCACTTGCAAAAC-3′; TcLOX2-F, 5′-TTAT**CTGCAG**CATGTTGAAGCCCCAAATTC-3′ (*Pst*I restriction site in bold); TcLOX1-R, 5′-AATA**GAATTC**GCAACCAAAATGTGCAC-3′; TcLOX2-R, 5′-AATA**GAATTC**CTGTAATTAGATGGAGATGC-3′(*Eco*RI restriction site in bold). The amplified product was digested by *PstI/EcoRI* and cloned into the pRSET-A expression vector (Invitrogen) fused to an amino-terminal histidine tag, and expressed in *Escherichia coli* BL-21 under the control of an isopropylthio-β-galactoside inducible promoter.

Heterologous Expression of *TcLOX1* and *TcLOX2* in *E. coli*

E. coli cells transformed with the recombinant and the empty constructs, were grown to an OD_{600} of 0.6 and then induced with 1 mM IPTG at 18 °C for 20 h. After harvesting by centrifugation, the cells were resuspended in 1 ml resuspension buffer (0.05 M Tris/HCl pH 8, 0.01 % β-mecaptoethanol) and 10 µL Lysozyme (100 mg/mL), disrupted by sonification (6 × for 10 s), and centrifuged at 13,000 *g* for 10 min at 4 °C. His-tagged proteins were purified with Ni^{2+}–NTA column (Qiagen) according to the manufacturer's protocol. Enzyme induction and purification success were checked by SDS-PAGE and with a soybean LOX standard.

In Vitro Enzymatic Activity Assay

His-tag purified proteins were assayed for lipoxygenase activity [17, 18]. The assay consisted of: 200 µL eluted protein (ca. 1 mg/mL) in 0.05 M Tris/HCl pH 8, 0.01 % β-mercaptoethanol, 200 mM imidazole, 700 µL 0.1 M

Table 1 Relative gene expression of *LOX* ESTs compared to *TcCDS* and between tissues

Identifier	RGE-CDS[a]	RGE-tissues (%)[b]						
	(%)	Trichomes	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7
TcCDS	**100**	**100.0 ± 10.3**	16.7 ± 1.5	8.0 ± 0.5	6.3 ± 1.6	4.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
Ct938[c]	56	**100.0 ± 9.8**	9.8 ± 0.6	7.2 ± 0.7	4.2 ± 0.7	3.1 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
Ct20675[c]	32	**100.0 ± 8.8**	11.0 ± 0.2	7.5 ± 0.5	6.1 ± 0.8	3.4 ± 0.0	1.0 ± 0.1	1.2 ± 0.2
Ct425[c]	18	**100.0 ± 14.2**	7.5 ± 0.6	7.4 ± 1.0	6.9 ± 1.0	1.5 ± 0.0	0.6 ± 0.0	1.2 ± 0.1
Ct4895[c]	12	**100.0 ± 9.8**	12.6 ± 1.0	5.0 ± 0.5	3.8 ± 0.6	3.1 ± 0.2	0.8 ± 0.0	0.2 ± 0.0
Ct20982[c]	9.2	**100.0 ± 15.1**	34.4 ± 1.5	13.2 ± 1.6	12.9 ± 2.0	4.1 ± 0.3	3.6 ± 0.7	2.4 ± 0.3
Ct2442[c]	7.9	**100.0 ± 6.9**	10.2 ± 0.8	11.6 ± 1.5	10.1 ± 1.6	0.0 ± 0.0	0.8 ± 0.0	1.7 ± 0.4
Ct5842	0.003	**100.0 ± 8.3**	16.1 ± 0.2	9.9 ± 2.1	7.8 ± 0.1	2.2 ± 1.2	1.7 ± 0.1	3.6 ± 0.2
Ct15096	1.0	0.8 ± 0.0	**100.0 ± 3.9**	44.7 ± 3.7	25.2 ± 4.1	14.5 ± 1.1	26.7 ± 1.2	22.8 ± 2.8
Ct1737	0.11	1.9 ± 0.6	**100.0 ± 4.4**	55.0 ± 5.9	60.6 ± 8.6	26.7 ± 0.3	50.9 ± 3.5	50.9 ± 3.5
Ct4483	0.02	2.5 ± 0.7	**100.0 ± 3.4**	29.4 ± 0.3	38.0 ± 3.9	75.5 ± 3.0	41.3 ± 6.9	60.8 ± 15.0
Ct20867	2.1	26.3 ± 0.8	82.4 ± 2.8	**100.0 ± 3.4**	56.7 ± 3.6	36.1 ± 2.3	31.8 ± 1.2	88.9 ± 2.2
Ct22733	2.1	39.4 ± 1.4	93.0 ± 0.5	**100.0 ± 1.0**	77.8 ± 11.4	39.3 ± 2.3	40.6 ± 0.4	88.0 ± 2.2
Ct19896	1.3	27.7 ± 3.0	54.8 ± 5.6	**100.0 ± 3.4**	56.4 ± 0.6	16.1 ± 1.1	16.9 ± 3.5	50.9 ± 3.5
Ct2382	21	1.5 ± 0.0	64.3 ± 3.2	74.5 ± 5.1	92.8 ± 14.5	82.0 ± 1.6	74.9 ± 8.4	**100.0 ± 7.3**
Ct12107	9.2	0.5 ± 0.0	47.4 ± 1.4	46.8 ± 3.7	77.6 ± 9.5	36.6 ± 1.3	40.3 ± 1.8	**100.0 ± 7.3**
Ct22446	5.3	49.5 ± 5.3	9.2 ± 0.2	10.0 ± 0.3	20.2 ± 2.1	38.4 ± 3.6	34.2 ± 1.0	**100.0 ± 10.3**
Ct22571	3.7	59.8 ± 9.3	20.8 ± 0.7	14.5 ± 0.0	25.5 ± 2.6	46.3 ± 4.8	45.6 ± 1.1	**100.0 ± 11.2**
Ct107	3.4	38.5 ± 2.3	13.2 ± 1.3	10.3 ± 1.1	18.5 ± 3.2	32.5 ± 2.4	28.4 ± 1.7	**100.0 ± 7.3**
Ct366	2.8	2.2 ± 0.3	71.6 ± 4.9	24.4 ± 3.0	26.5 ± 3.4	46.4 ± 1.1	67.7 ± 0.7	**100.0 ± 9.8**
Ct2729	1.0	1.2 ± 0.4	63.8 ± 11.5	15.8 ± 1.8	22.6 ± 0.0	31.9 ± 3.3	42.5 ± 0.8	**100.0 ± 1.5**
Ct24032	0.63	1.4 ± 0.1	43.2 ± 1.5	18.7 ± 1.4	26.8 ± 1.4	31.9 ± 0.9	24.8 ± 0.4	**100.0 ± 7.3**
Ct26038	0.54	1.2 ± 0.3	32.7 ± 2.9	19.5 ± 1.1	22.5 ± 0.3	19.8 ± 2.0	27.3 ± 1.6	**100.0 ± 1.5**
Ct26547	0.34	1.6 ± 0.3	38.4 ± 16.9	18.6 ± 1.8	22.9 ± 1.9	36.0 ± 4.6	28.2 ± 2.5	**100.0 ± 5.9**
Ct26290	0.02	0.5 ± 0.1	84.0 ± 7.0	44.8 ± 1.3	61.2 ± 1.2	75.7 ± 38.3	52.3 ± 6.1	**100.0 ± 11.2**
Ct25085	0.02	2.3 ± 0.1	56.1 ± 1.6	34.0 ± 1.0	56.2 ± 9.1	74.9 ± 7.0	51.5 ± 14.5	**100.0 ± 3.4**

Errors represent the SD between two technical replicates

Values in boldface represent the tissue (trichome or ovary developmental stage) at which the RGE was the highest

[a] RGE-CDS is relative gene expression of *LOX* genes relative to *CDS* (17.3 cycles), and were calculated as the ratio of the maximum *LOX* and *CDS* gene expression (linear scale) as measured by RT-qPCR multiplied by 100 %

[b] RGE-tissues is relative gene expression relative to the different tissues/development (trichomes and flowering stages) for the same gene

[c] LOX ESTs with a *TcCDS*-like expression profile have highest expression in the trichomes (100 %). Single underlined ESTs represent fragments of *TcLOX1* cDNA and double underlined ESTs of *TcLOX2* cDNA

Tris/HCl assay buffer (pH 7.5), 100 µL H_2O_2 (3 % in assay buffer), and 5 µL linolenic acid (50 mM in 100 % EtOH). Assays without enzyme and with 200 µg soybean lipoxygenase (Fluka 62340) were used as negative and positive controls respectively. The assays were incubated for 30 min at room temperature, and then filtered through 0.2 µm inorganic membrane filters (RC4, Sartorius, Germany) before HPLC analysis.

HPLC Analysis of In Vitro Assays

HPLC analysis was performed using a Waters Alliance 2795 HPLC connected to a Waters 2996 PDA detector (Waters, MS technologies, UK). The column used was a C18 Luna analytical column (4.6 × 150 mm; Phenomenex, USA) attached with a C18 pre-column (2.0 × 4 mm; Phenomenex, USA). Degassed eluent A (Water with 0.1 % HAc) and eluent B (Methanol with 0.1 % HAc) were pumped at 1 mL min^{-1} into the HPLC system. The gradient started at 10 % B and increased linearly to 100 % B in 23 min. Then the column was washed and equilibrated for 7 min before the next injection. The injection volume was 50 µL. An authentic standard of 13 (*S*)-hydroperoxy-9(*Z*),11-(*E*),15-(*Z*)-octadecatrienoic acid (13-(*S*)-HPOT) was purchased from Loradan (Malmo, Sweden).

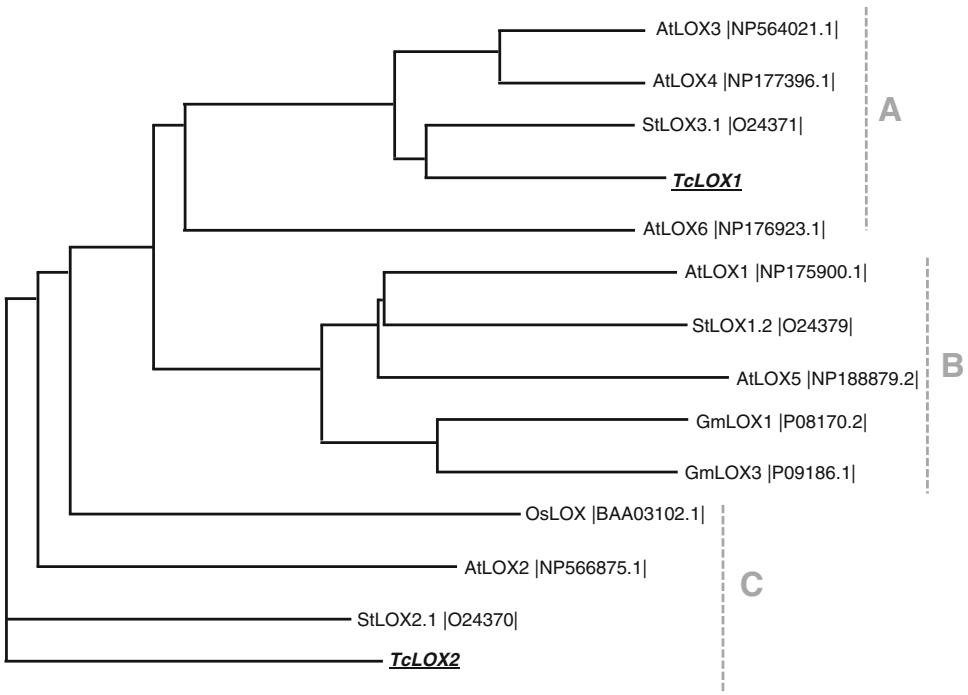

Fig. 3 Phylogenetic tree constructed by pairwise comparison of the deduced amino acid sequences of the two putative pyrethrum LOX proteins (TcLOX1 and TcLOX2) and other biochemically characterized plant LOX. The tree was constructed by Neighbour-Joining method using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The species abbreviations are At, *Arabidopsis thaliana*; Gm, *Glycine max; St, Solanum tuberosum; Os, Oryza sativa*

Results

Isolation and Selection of *LOX* EST Candidates

To isolate LOX genes involved in pyrethrin biosynthesis, the pyrethrum annotated EST contig library was subjected to a keyword search using LOX or lipoxygenase as keywords. Among the 27,314 high-quality contigs, 25 EST sequences with homology to LOX genes were retrieved, but none were full length (Table S1, supplemental data). Given our previous observations on the similar transcriptional profiles of *TcCDS* and *TcGLIP* during flower development and the respective high and low expression in trichomes [13], we hypothesized that EST contigs showing a similar expression pattern may be involved in pyrethrin biosynthesis. Among the 25 candidate contigs, six, named Ct938, Ct20675, Ct425, Ct4895, Ct20982, Ct2442 and Ct5842, showed a relative gene expression (RGE) profile similar to that of *TcCDS*, with highest expression in trichomes, and intermediate expression during stages 2–5 followed by a pronounced decreased in stages 6 and 7. Ct5842 was not investigated further due to a very RGE of 0.003 %. Ct15096 followed a *TcGLIP*-like pattern of expression, but this lead was not followed either, because of its RGE of 1.0 % relative to the expression level of *TcCDS* (Table 1, Column RGE-CDS). All others had highly deviant patterns of accumulation. The six EST contigs with an expression similar to *TcCDS* were selected as genes possibly involved in pyrethrin biosynthesis. After RACE amplification of the 3′ and 5′ ends, assembly of the six selected ESTs resulted in two complete, full-length genes, *TcLOX1* and *TcLOX2*.

Phylogenetic Analysis of *TcLOX1* and *TcLOX2*

A phylogenetic analysis was performed in which the putative pyrethrum lipoxygenase sequences (*TcLOX1* and *TcLOX2*) were compared with those of 12 biochemically characterized plant lipoxygenases [19]. The phylogenetic tree (Fig. 3) displayed three main clusters, designated as A, B, and C. All lipoxygenases in cluster A are 13-lipoxygenases with a plastidic N-terminal localization peptide [19, 20]. Two cytoplasmic localized lipoxygenases with 13 (*GmLOX1*) or with a dual 9/13 specificity (*GmLOX3*) [21, 22], and the 9-lipoxygenase representatives with either a cytoplasmic (*AtLOX1* and *StLOX1.2*) or plastidic localization (*AtLOX5*) group in cluster B [19, 20]. The remaining 13-lipoxygenases with plastidic localization (*OsLOX*, *StLOX2.1 and AtLOX2*) form Cluster C [19, 20, 23]. *TcLOX1*, shows a close sequence similarity with the genes of cluster A, which have been characterized as 13-lipoxygenases with chloroplast localization. Within this cluster pyrethrum *TcLOX1* shows a significant similarity (75 %) to the *Solanum tuberosum LOX3.1* [X96406 (H3)], [20]. *TcLOX2*, on the other hand, falls into Cluster C, with a 60 % similarity to the chloroplast localized *Solanum tuberosum LOX2.1 (StLOX2.1),* and sharing only 40 % similarity with the *TcLOX1* (Fig. 4).

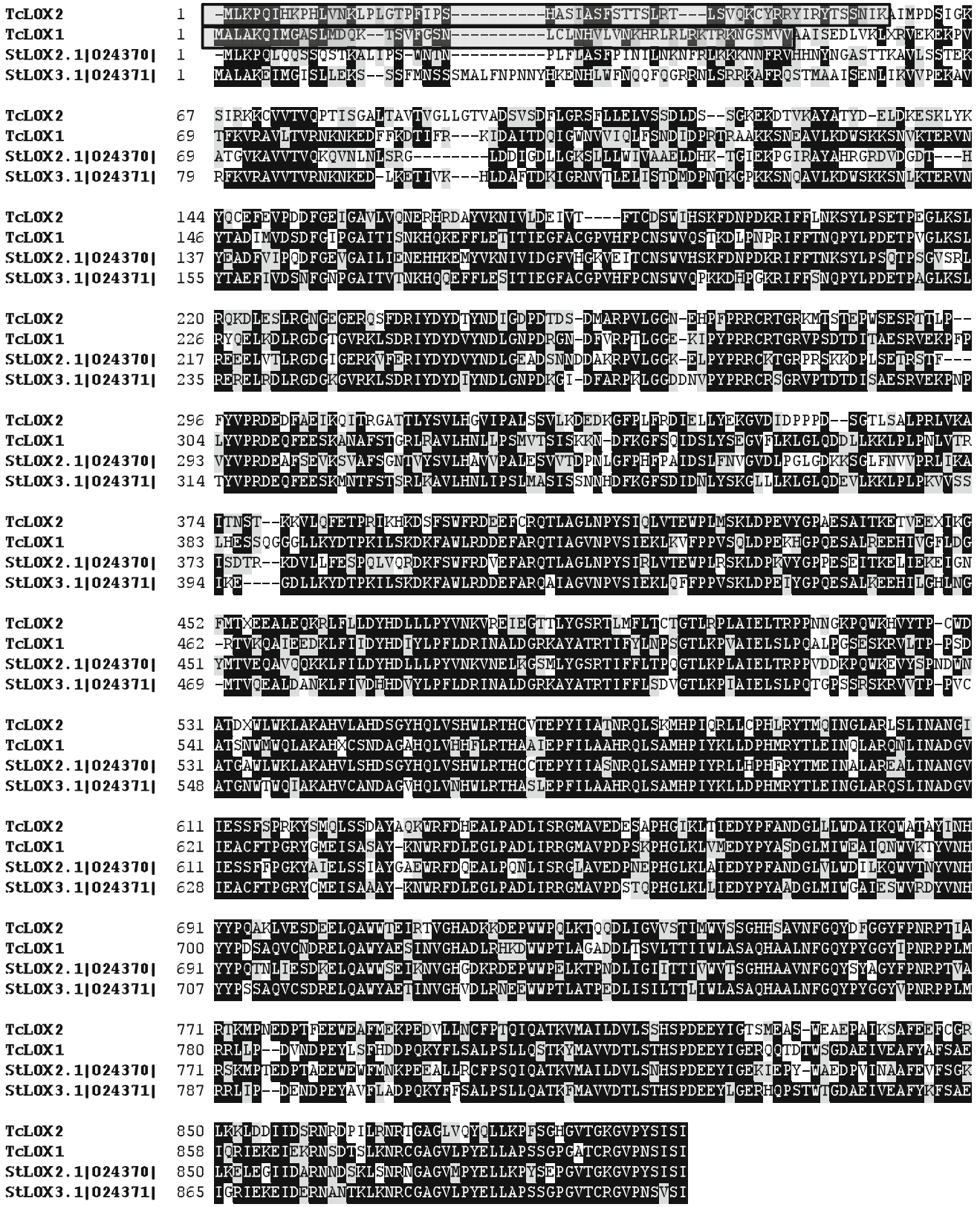

Fig. 4 Sequence alignment based on the deduced amino acid sequence of the two pyrethrum LOX genes (*TcLOX1* and *TcLOX2*), and the two potato LOX genes (*StLOX2.1* and *StLOX3.1*) showing the chloroplast targeting peptide (in the *box*) on both pyrethrum LOX genes as predicted by the ChloroP1.1 prediction server (http://www.cbs.dtu.dk/services/ChloroP/). The alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2)

Functional Characterization of Recombinant TcLOX1

The full-length *TcLOX1* cDNA consisted of 2724 bp encoding a polypeptide of 907 amino acids (Figure S1, supplemental data), with a calculated molecular mass of 102.5 kDa and a predicted iso-electric point of 7.2. *TcLOX2* consisted of a 2700 bp cDNA sequence encoding an 899 amino acids polypeptide (Figure S2, supplemental data). In addition and as reported for other *13-LOX* genes from plants [19], they both contained a chloroplast targeting peptide, as predicted by the ChloroP1.1 prediction server (http://www.cbs.dtu.dk/services/ChloroP/) (Fig. 4, grey highlighted area).

To determine whether the cDNAs encoded functional enzymes, an empty pRSET-A vector and two recombinant ones containing the full-length *TcLOX* cDNAs were transformed into *E. coli* and purified. The HPLC chromatogram of the products of the enzymatic assay performed with protein purified from cells transformed with *TcLOX1* showed a peak (Fig. 5b, arrow), that was not detected in assays performed with protein derived from cells transformed with the control plasmid (Fig. 5a) and *TcLOX2* (data not shown). TcLOX1 activity was confirmed by running the same enzymatic assay with a non-recombinant purified soybean LOX protein sample. The assay showed a reaction product co-eluting with the reaction product of the recombinant pyrethrum LOX (Fig. 5c). Comparison of the RT and UV spectrum with that of a 13-hydroperoxylinolenic acid authentic standard (14-1803-2, Loradan) demonstrated that pyrethrum TcLOX1 and non-recombinant soybean LOX are lipoxygenases (Fig. 5d).

Tissue Specificity and Developmental Pattern of *TcLOX* Gene Expression

Besides the initial RTqPCR carried out on cDNA templates isolated from flowers of different developmental stages and from stage 4 trichomes, the expression of *TcLOX1* was also assessed using cDNA samples from leaves, seedlings and trichomes isolated from stage 3 ovaries.

Comparison of the relative gene expression (RGE) of *TcLOX1* with *TcCDS*, [11], reveals that both genes consistently show a similar pattern of expression along flower development. That is intermediate expression in flowers of the early developmental stages (Fig. 6a, stages 2–5), to considerably lower expression in later stages (Fig. 6a, stages 6 and 7). Both genes are expressed considerably in isolated trichomes, higher than in intact ovaries (ovaries with trichomes) and leaves. The expression of both genes, furthermore, was completely absent in seedlings that are devoid of glandular trichomes (Fig. 6b).

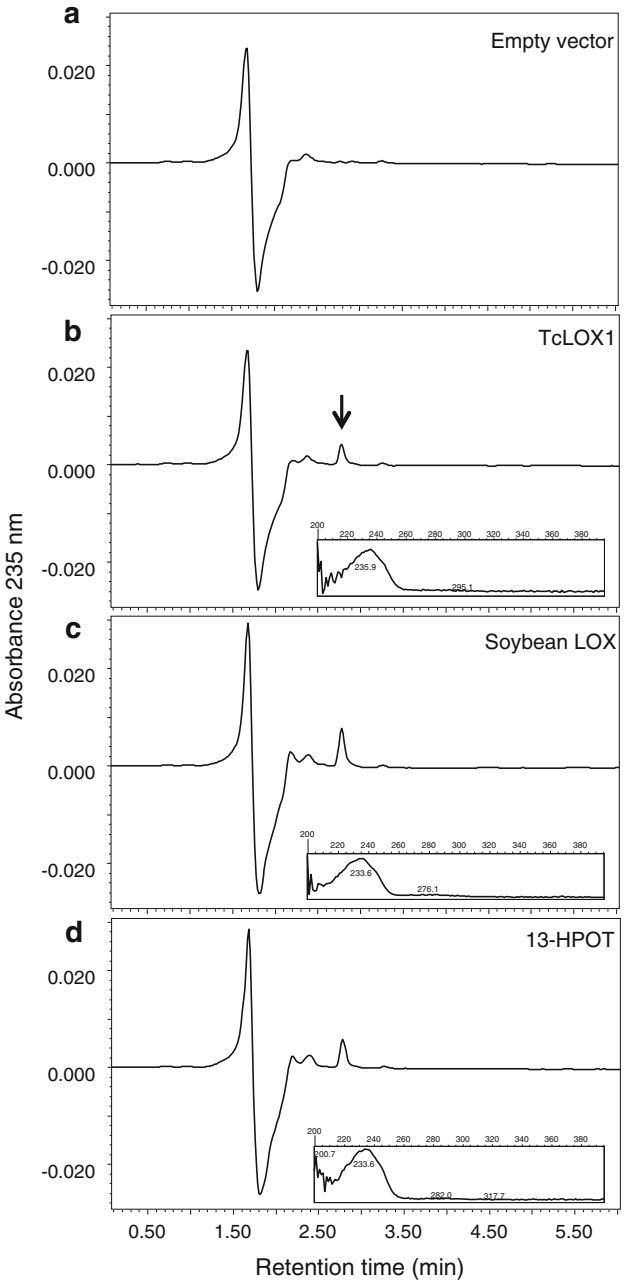

Fig. 5 HPLC chromatograms and PDA spectra of hydroxylinolenic acid (*arrow*) of the enzyme assay performed with the empty vector control (**a**), purified recombinant TcLOX1 protein (**b**), non-recombinant soybean LOX (**c**), and the 13-hydroperoxylinolenic acid standard (**d**)

Discussion

Matsuda et al. [2] demonstrated using ^{13}C-glucose as a precursor in pyrethrum seedlings that the alcohol moiety of pyrethrins most likely derives from linolenic acid through the oxylipin pathway (Figs. 1, 2). The oxylipin pathway is best known to lead to the formation of jasmonic acid and

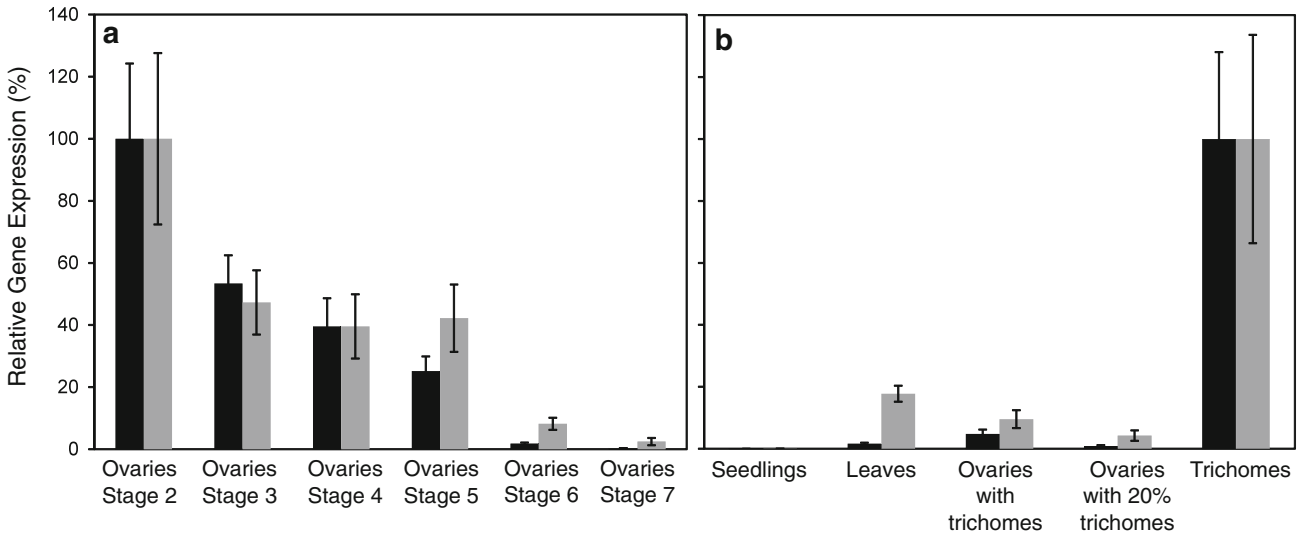

Fig. 6 Relative gene expression of *TcCDS* (*black bars*) and *TcLOX1* (*grey bars*) in ovaries isolated from flowers in different developmental stages (**a**); and isolated glandular trichomes, ovaries with trichomes, ovaries with 80 % fewer trichomes, leaves and seedlings (**b**) after normalization against the *GAPDH* gene

has been shown to start with the release of linolenic acid (1) from chloroplast cell membranes, which is subsequently oxidized at position C13 by a lipoxygenase. The resulting 13-hydroperoxylinolenic acid (13-HPOT (2)), is converted to JA (4) through reduction and a series of β-oxydations [8, 24, 25]. Given the high resemblance of jasmolone, pyrethrolone and cinerolone to jasmonic acid, Matsuda hypothesized that if they are indeed synthesized through the oxylipin pathway, a chloroplast located lipoxygenase capable of catalyzing a C13 peroxidation of the linolenic acid substrate would be required for the biosynthesis of the alcohol moiety of pyrethrins. In our previous study, we showed that the monoterpene acid moiety of pyrethrin biosynthesis starts in the trichomes and is transcriptionally regulated along flower development [13]. In the early flower developmental stages (stages 1–5) when pyrethrins accumulate the expression of *TcCDS*, which is responsible for catalyzing the first step of the formation of the acid moiety of pyrethrins [11], is high. In the later stages (stages 6 and 7) when pyrethrin content is no longer increasing, the transcript level of *TcCDS* is very low (<5 % of initial values). This is also true for *TcGLIP,* which is responsible for the final esterification step in pyrethrin biosynthesis although the decrease is slightly less pronounced (<20 % of initial values)[13].To identify and isolate a lipoxygenase gene potentially involved in the first step towards the biosynthesis of the alcohol moiety of pyrethrins, we retrieved 25 putative candidate EST contigs from a provisionally annotated EST library by using LOX or Lipoxygenase as keyword. Subsequently, expression profiling was done, resulting in the selection of six candidate EST contigs showing the pattern and level of expression most similar to *TcCDS*. A pattern of expression similar to *TcGLIP* was recognized in the EST Ct15096, but not further investigated, because of low maximum expression relative to *TcCDS* (1.0 %, Table 1), making it a less likely candidate in the pathway. After RACE amplification of these six selected ESTs, they assembled into only two full-length genes, named *TcLOX1* and *TcLOX2*. The cDNAs of both *TcLOX* genes were expressed in *E.coli* but only TcLOX1 was catalytically active as a lipoxygenase. TcLOX2 did not yield any product, which points to a possibly more labile nature of this enzyme, similar to what has been reported for AtLOX2, which failed to produce measurable amounts of products after the cells were sonicated [19], or a different optimal pH. Some lipoxygenases have been shown to have their optimal activity at pH values of 5.5 [23] rather than at neutral pH values (7.5) as used in this study.

Based on the primary structure of the enzyme, the positional specificity and subcellular targeting of plant lipoxygenases can be predicted with high confidence [26, 27]. Our phylogenetic analysis clearly shows that both TcLOXes group together with other biochemically-characterized 13-lipoxygenases, which have also been reported to contain an N-terminal localization peptide that directs them to the chloroplast [19, 20, 23]. However, these 13-lipoxygenases only share a 40 % similarity and therefore cluster into two distant groups (A and C, respectively). Interestingly, although according to the phylogenetic relationship both *TcLOXes* are classified as type-2 13-LOXes, TcLOX1 clusters closer to the potato LOX3 (*StLOX3.1*) and the Arabidopsis LOX3 and LOX4, whereas TcLOX2 shows closer sequence similarity to the potato LOX2

(*StLOX2.1*). Potato LOX2 is involved in de-novo wound induced JA accumulation, whereas potato LOX3 has been suggested to be implicated in maintaining basal JA levels hence, being present at constant level in both non-wounded and wounded tissues [20]. Arabidopsis LOX3 and LOX4 have been demonstrated to play important roles in developmental processes such as plant fertility, which also suggests a developmental rather than a wound-induced regulation, and this role may also be manifest in pyrethrum trichomes [28]. Although in pyrethrum seedlings wounding resulted in an enhanced content of pyrethrins [29], in both flowers and leaves, biosynthesis is developmentally regulated [13]. Nevertheless, both genes may potentially work in parallel to supply the required precursors for pyrethrin biosynthesis in response to development and/or wounding.

One of the most fascinating features of the two lipoxygenases is that both *TcLOX*1 and *TcLOX2* display a very similar and dominant expression in trichomes. If this is significant of either of these genes to be involved in pyrethrin biosynthesis, this would imply that also the rethrolone or an earlier precursor may be exported from glandular trichomes into the pericarp, where TcGLIP catalyzes the esterification of the acid and the rethrolone moieties. Our results may thus indicate that potentially similar mechanisms of precursor export from trichomes to pericarp could be operating for the alcohol moiety. However, an alternative production by for example the enzyme encoded by Ct15096 that followed a *TcGLIP* expression pattern, in the tissues that synthesize *TcGLIP* cannot be ruled out at this point [13]. To prove either way, it will be necessary, for example, to generate transgenic pyrethrum with down-regulated expression of these genes to observe any effects on the accumulation of pyrethrins and free chrysanthemic acid [30].

Acknowledgments We thank Bas te Lintel Hekkert for his contribution to the construction of the pyrethrum EST contig library. We also thank Harry Jonker for his assistance with the HPLC measurements, Jos Molthoff for his assistance with the RTqPCR experiments, and Marcel Dicke for critically reviewing the article. This research was supported by Technology Top Institute Green Genetics (Stichting TTI Groene Genetica) of the Netherlands (Grant No. 1C001RP).

References

1. Crombie L (1995) Chemistry of pyrethrins. In: Casida JE, Quistad GB (eds) Pyrethrum flowers: production, chemistry, toxicology, and uses. Oxford University Press, New York, pp 123–193
2. Matsuda K, Kikuta Y, Haba A, Nakayama K, Katsuda Y et al (2005) Biosynthesis of pyrethrin I in seedlings of *Chrysanthemum cinerariaefolium*. Phytochemistry 66:1529–1535
3. Browse J (2005) Jasmonate: an oxylipin signal with many roles in plants. In: Litwack G (ed) Plant Hormones, pp 431–456
4. Vellosillo T, Martinez M, Lopez MA, Vicente J, Cascon T et al (2007) Oxylipins produced by the 9-lipoxygenase pathway in Arabidopsis regulate lateral root development and defense responses through a specific signaling cascade. Plant Cell 19:831–846
5. Vick BA, Zimmerman DC (1983) The biosynthesis of jasmonic acid. A physiological role for plant lipoxygenase. Biochem Biophys Res Commun 111:470–477
6. Howe GA, Jander G (2008) Plant immunity to insect herbivores. Ann Rev Plant Biol 59:41–66
7. Prost I, Dhondt S, Rothe G, Vicente J, Rodriguez MJ et al (2005) Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol 139:1902–1913
8. Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot 100:681–697
9. Feussner I, Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol 53:275–297
10. Liavonchanka A, Feussner N (2006) Lipoxygenases: occurrence, functions and catalysis. J Plant Physiol 163:348–357
11. Rivera SB, Swedlund BD, King GJ, Bell RN, Hussey CE et al (2001) Chrysanthemyl diphosphate synthase: isolation of the gene and characterization of the recombinant non-head-to-tail monoterpene synthase from *Chrysanthemum cinerariaefolium*. Proc Natl Acad Sci USA 98:4373–4378
12. Kikuta Y, Ueda H, Takahashi M, Mitsumori T, Yamada G et al (2012) Identification and characterization of a GDSL lipase-like protein that catalyzes the ester-forming reaction for pyrethrin biosynthesis in *Tanacetum cinerariifolium*—a new target for plant protection. Plant J 71:183–193
13. Ramirez AM, Stoopen G, Menzel TR, Gols R, Bouwmeester H et al (2012) Bidirectional secretions from glandular trichomes of pyrethrum enable immunization of seedlings. Plant Cell 10: 4252–4265
14. Li J, Yin LY, Jongsma MA, Wang CY (2011) Effects of light, hydropriming and abiotic stress on seed germination, and shoot and root growth of pyrethrum (*Tanacetum cinerariifolium*). Ind Crops Prod 34:1543–1549
15. Rothberg JM, Leamon JH (2008) The development and impact of 454 sequencing. Nat Biotechnol 26:1117–1124
16. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25:402–408
17. Santino A, Iannacone R, Hughes R, Casey R, Mita G (2005) Cloning and characterisation of an almond 9-lipoxygenase expressed early during seed development. Plant Sci 168:699–706
18. Wang R, Shen WB, Liu LL, Jiang L, Liu YQ et al (2008) A novel lipoxygenase gene from developing rice seeds confers dual position specificity and responds to wounding and insect attack. Plant Mol Biol 66:401–414
19. Bannenberg G, Martinez M, Hamberg M, Castresana C (2009) Diversity of the enzymatic activity in the lipoxygenase gene family of *Arabidopsis thaliana*. Lipids 44:85–95
20. Royo J, Vancanneyt G, Perez AG, Sanz C, Stormann K et al (1996) Characterization of three potato lipoxygenases with distinct enzymatic activities and different organ-specific and wound-regulated expression patterns. J Biol Chem 271:21012–21019
21. Coffa G, Imber AN, Maguire BC, Laxmikanthan G, Schneider C et al (2005) On the relationships of substrate orientation, hydrogen abstraction, and product stereochemistry in single and double dioxygenations by soybean lipoxygenase-1 and its Ala542Gly mutant. J Biol Chem 280:38756–38766
22. Yenofsky RL, Fine M, Liu C (1988) Isolation and characterization of a soybean (*Glycine max*) lipoxygenase-3 gene. Mol Gen Genet 211:215–222

23. Peng YL, Shirano Y, Ohta H, Hibino T, Tanaka K et al (1994) A novel lipoxygenase from rice. Primary structure and specific expression upon incompatible infection with rice blast fungus. J Biol Chem 269:3755–3761
24. Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc Natl Acad Sci USA 92:8675–8679
25. Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. Ann Rev Plant Physiol Plant Mol Biol 48:355–381
26. Hornung E, Walther M, Kuhn H, Feussner I (1999) Conversion of cucumber linoleate 13-lipoxygenase to a 9-lipoxygenating species by site-directed mutagenesis. Proc Natl Acad Sci USA 96:4192–4197
27. Hughes RK, West SI, Hornostaj AR, Lawson DM, Fairhurst SA et al (2001) Probing a novel potato lipoxygenase with dual positional specificity reveals primary determinants of substrate binding and requirements for a surface hydrophobic loop and has implications for the role of lipoxygenases in tubers. Biochem J 353:345–355
28. Caldelary D, Wang G, Fermer EE, Dong X (2011) Arabidopsis lox3 lox4 double mutants are male sterile and defective in global proliferative arrest. Plant Mol Biol 75(1–2):25–33
29. Ueda H, Matsuda K (2011) VOC-mediated within-plant communications and nonvolatile systemic signals upregulate pyrethrin biosynthesis in wounded seedlings of *Chrysanthemum cinerariaefolium*. J Plant Int 6:89–91
30. Mao J, Cao LY, Kong LF, Jongsma MA, Wang CY (2013) An Agrobacterium-mediated transformation system of pyrethrum (*Tanacetum cinerariifolium*) based on leaf explants. Sci Hortic 150:130–134