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Cloning and expression of sesquiterpene synthase genes from lettuce (*Lactuca sativa* L.)

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Abstract

Sesquiterpenoid lactones (SLs) from lettuce (*Lactuca sativa* L.) include constitutive components of latex such as lactucin and the induced phytoalexin, lettucenin A. A redundant primer strategy was used to recover two full length cDNA clones (*LTC1* and *LTC2*) encoding sesquiterpene synthases from a cDNA library derived from seedlings with the red spot disorder, which accumulate phytoalexins. Recombinant enzymes produced from *LTC1* and *LTC2* in *Escherichia coli* catalysed the cyclisation of farnesyl diphosphate to germacrene A, potentially an early step in the biosynthesis of SLs. RT-PCR analysis showed *LTC1* and *LTC2* were expressed constitutively in roots, hypocotyls and true leaves but not in cotyledons. Expression in cotyledons was induced by challenge with the downy mildew pathogen *Bremia lactucae* in the disease resistant cultivar Diana. Southern hybridisation experiments showed that *LTC1* and *LTC2* were not part of a multigene family. The germacrene A synthases provide targets for modified expression to generate beneficial modifications to the SL profile in lettuce. © 2002 Published by Elsevier Science Ltd.

Keywords: *Lactuca sativa*; Compositae; Sesquiterpene lactones; Phytoalexin; Lettucenin A; Germacrene A synthase; Gene expression

1. Introduction

Like other members of the compositae, species of *Lactuca* contain several sesquiterpenoid lactones (SLs) (Pyrek, 1977; Rees and Harborne, 1985; Picman, 1986; Price et al., 1990). For example, lettuce (*Lactuca sativa*) has been reported to produce constitutive SLs such as lactucin, which accumulate within laticifers as components of latex, and also the phytoalexins lettucenin A and costunolide which are induced to accumulate following microbial challenge (Burnett et al., 1978; Takasugi et al., 1985; Picman, 1986; Bennett et al., 1994; Grayer and Harborne, 1994). Metabolite profiling of SLs from lettuce latex led to the recent identification of novel 15-oxalyl and 8-sulphate conjugates of the guaianolide SLs lactucin, deoxylactucin and lactucopicrin (Sessa et al., 2000). The presence of SLs in latex, which is released from damaged laticifers, is thought to contribute to its analgesic, antitussive and sedative properties (Mahmoud et al., 1986; Gromek et al., 1992). The constitutive SLs may also have a role as antifeedants (Rees and Harborne, 1985; Dussourd and Denno, 1994). The SLs lactucin, 8-deoxylactucin and lactucopicrin are also intensely bitter and their presence in salad lettuce and the closely related chicory (*Cichorium intybus* L.) has considerable economic impact (Price et al., 1990).

Recent progress in understanding the biosynthesis of sesquiterpenoids has been achieved by cloning genes encoding key enzymes, most notably terpene synthases [cyclases] (Chappell, 1995; Bohlmann et al., 1998; Davis et al., 1998). A proposal for the biosynthesis of SLs in lettuce is outline in Fig. 1. An intriguing feature is the ability of sesquiterpene synthases to generate compounds with different skeleta. The cyclases typically produce single products from farnesyl diphosphate (FPP), but in some cases (e.g. δ-selinene and γ-humulene synthases from *Abies grandis*) multiple products may be formed (Steele et al., 1998). A cyclase from *Artemisia*

Abbreviations: FPP, farnesyl diphosphate; TEAS, tobacco *epi*-aristolochene synthase; GC–MS, gas chromatography–mass spectrometry; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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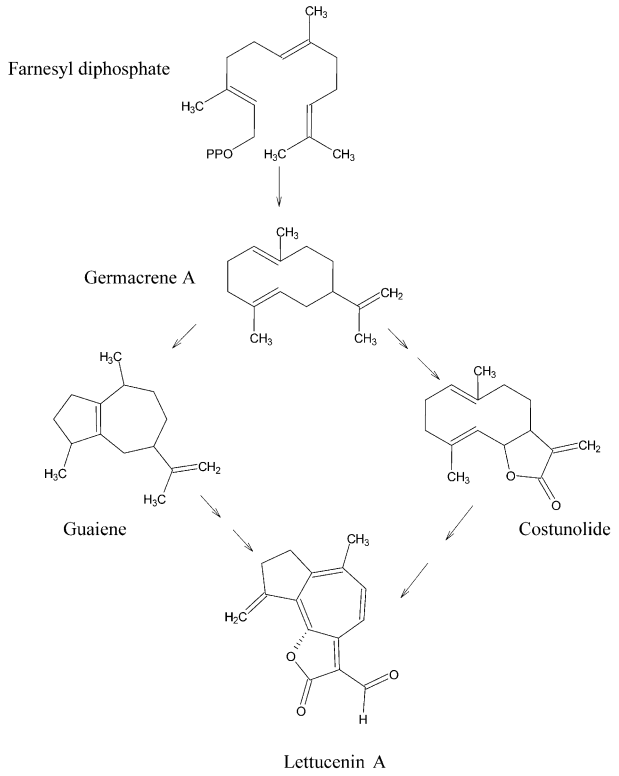

Fig. 1. Alternative routes for the biosynthesis of the guaianolide phytoalexin, lettucenin A. Steps proposed to involve several enzymes are indicated by double arrows.

annua even generates an hydroxylated product, 8-epicedrol (Hua and Matsuda, 1999; Mercke et al., 1999). In lettuce, it is not known if the germacranolide and guaianolide SLs are derived from the products of the same or different sesquiterpene synthases. It is possible that a single enzyme is involved, with guaiene arising from rearrangements of a primary germacrene product generated from FPP within the active site of the enzyme. Guaiene is one of the multiple products generated by the cyclase from *Abies* (Steele et al., 1998). However, in work on chicory (which is closely related to *Lactuca* species), de Kraker et al. (1998, 2001) have suggested that oxidation of germacrene A leads to germacranolide production prior to secondary ring closure to give guaianolides. Their hypothesis is based primarily on the finding of germacrene A synthase, but not guaiene synthase activity in tissue extracts.

Here, we report the cloning of sesquiterpene synthase genes from lettuce. The initial approach adopted was to use PCR to amplify the genes from a cDNA library derived from lettuce seedlings with the physiological red spot disorder; since such seedlings accumulate high levels of the guaianolide phytoalexin lettucenin A during development (Bestwick et al., 1995). Two induced sesquiterpene synthases were cloned, expressed in *Escherichia coli* and found to produce germacrene A from FPP.

2. Results

2.1. Use of degenerate primers to clone synthases

Primer pairs for PCR amplification were developed from conserved regions found in the DNA sequences of sesquiterpene synthases located in databases at the start of this project. A cDNA library was prepared in λ GT11 from lettuce seedlings with the red spot disorder. A PCR product amplified from the library with the primers was cloned into pGEMT and sequenced. Considerable similarity was found between the sequence of the amplified fragment and various sesquiterpene synthases in databases.

The cDNA fragment was radio-labelled and hybridised at high stringency (0.1% SSC) to the cDNA library. Primary screening revealed 80 positive plaques. Two rounds of secondary screening resulted in selection of five potentially full length cDNA clones; these were sub-cloned into pBluescript and sequenced. One clone was unique, designated *LTC1*, and the other four were found to contain the same cDNA, designated *LTC2*.[1] The amino acid sequences predicted for LTC1 and LTC2 were 92% identical to each other. Sequence alignments with other known sesquiterpene cyclases with established enzyme activity are summarized in Fig. 2. The activities of similar proteins ranged from germacrene C synthase to other cyclases which produced bicyclic and hydroxylated products e.g. 8-epicedrol (Hua and Matsuda, 1999).

2.2. Expression of recombinant proteins and enzyme activity

Levels of expression of recombinant LTC1 and LTC2 in *Escherichia coli* from the pET28 vector were initially very low due to the presence of codons for arginine which are rarely used in *E. coli* and also occurred in tandem (e.g. positions 61 and 62 in LTC1, Fig. 2). The codon usage problems were overcome by the use of an *E. coli* strain containing pRIL that expresses corresponding tRNAs. Enhanced expression of sesquiterpene synthases was more apparent in extracts of *E. coli* harbouring *LTC2* than *LTC1* (Fig. 3).

Enzyme activity was examined by analysis of product formation after the addition of [1–^{3}H]- farnesyl diphosphate to protein extracts of *E. coli* expressing the cloned genes. Analysis of incorporated products by radio-TLC of hexane extracts is shown in Fig. 4. After scaling up reactions, products were identified by GC–MS. LTC2 generated a single product which was identified as germacrene A by comparison of its spectrum (Fig. 5) and retention index with library data (Adams, 1995), after

[1] Nucleotide sequences of the germacrene A synthases *LTC1* and *LTC2* are available from GenBank, accession numbers AF489964 and AF489965, respectively.

Fig. 2. Alignment of amino acid sequences of representative sesquiterpene synthases with confirmed enzymatic activity. Identical and similar amino acids are indicated by black and grey boxing respectively. Synthases shown in addition to LTC1 and LTC2, and GenBank accession numbers are; aris, *epi*-aristolochene synthase (Q40577, Starks et al., 1997); cad, δ-cadinene synthase (P93665, Davis et al., 1998); germ, germacrene C synthase (T06265, Colby et al., 1998) and ced, 8-epicedrol synthase (AAF80333, Hua and Matsuda, 1999; Mercke et al., 1999). Note that the degenerate primers used to amplify the lettuce synthases correspond to amino acids 22–33 and 500–510 in LTC1.

cool on-column injection. Additionally, at hot injection temperatures the characteristic Cope rearrangement of germacrene A to β-elemene was observed. Lower levels of incorporation were found with LTC1, from which the major product was again germacrene A. The additional minor peak of a more polar product detected by TLC of LTC1 reactions at 75–85 mm (Fig. 4) was not identified, but its polarity indicated that it was not a sesquiterpene hydrocarbon. Activities of both LTC1 and LTC2 were examined over the pH 5.6–9.0 range; the optimum incorporation for both enzymes was at pH7.0. At lower pHs LTC1 generated proportionately more of the polar product (as shown in Fig. 3), but pH did not affect product formation by LTC2. Like other sesquiterpene cyclases, LTC1 and LTC2 germacrene A synthases required the presence of Mg^{2+} (Bohlmann et al., 1998).

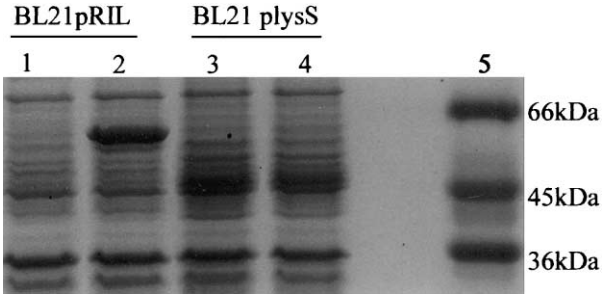

Fig. 3. Expression of LTC1 and LTC2 proteins from pET28 in *E. coli* strains BL21pRIL and BL21pLysS. Extracts were separated by SDS/PAGE. Lanes were loaded with, 1 and 3 Pet28 + LTC1; 2 and 4, Pet28 + LTC2, and 5, molecular weight markers. The strongly expressed, LTC2 synthase in lane 2 has a predicted MW of 64.2 kDa.

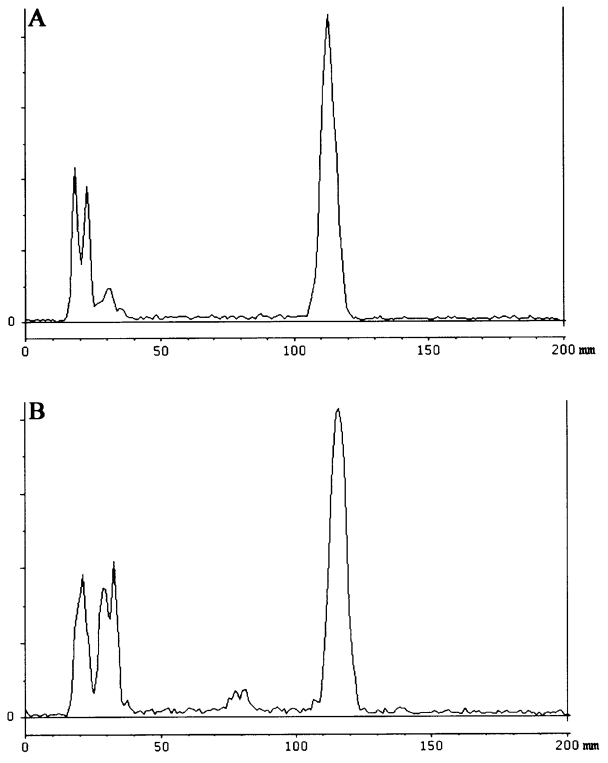

Fig. 4. Enzyme activity of LTC1 and LTC2 at pH 6.0. [1–^{3}H] farnesyl diphosphate was used as a substrate to examine catalytic activity in extracts of *E. coli* expressing the synthase proteins. Incorporation into one major sesquiterpene product was demonstrated by radio-TLC (as shown), which was identified as germacrene A by GC–MS. Analysis of chomatograms is shown for A, LTC2 and B, LTC1; samples were loaded at 20 mm. Peaks indicate detection of radioactivity on the TLC plate.

2.3. Expression of LTC1 and LTC2 is induced during challenge by Bremia lactucae

Expression of *LTC1* and *LTC2* was examined by RT-PCR using primer pairs designed to be specific for each cDNA. The two genes were found to be expressed constitutively in lettuce roots, hypocotyls and true leaves, but level of the *LTC1* transcript was much lower than *LTC2* in leaves (Fig. 6). Transcripts were not detected in cotyledons but they accumulated after challenge with

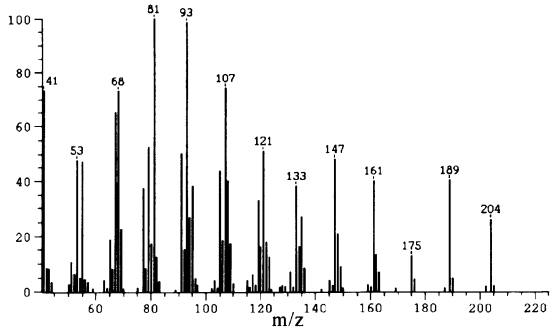

Fig. 5. GC–MS analysis (from cold injection) of germacrene A produced from farnesyl diphosphate by recombinant LTC2.

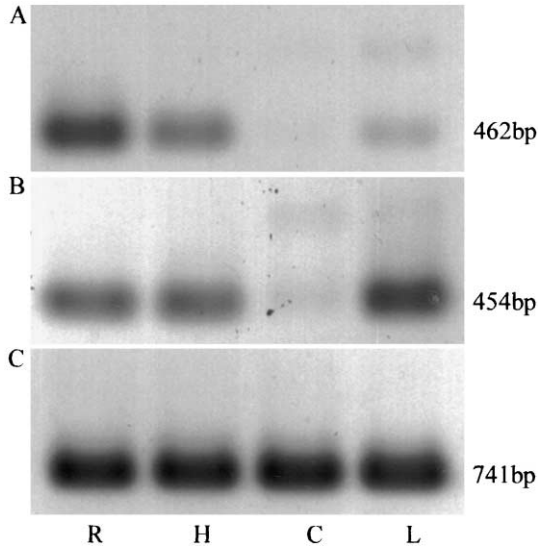

Fig. 6. RT-PCR analysis of gene expression in different plant tissues, A, *LTC1*; B, *LTC2* and C, *triosephosphate isomerase* as a constitutively expressed control. RNA was extracted from samples of R, root; H, hypocotyl; C, cotyledon and L, first true leaf. Lanes show PCR products separated on 1% agarose gels. Amplification products were: A 462 bp; B 454 bp and C 741 bp as expected from sequence data.

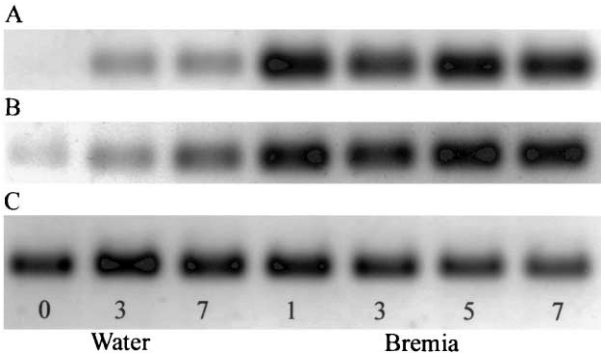

Fig. 7. The sesquiterpene synthases are induced by infection. Analysis of *LTC1* and *LTC2* expression in lettuce cotyledons by RT-PCR. A–C, induction of transcripts in cotyledons 0, 1, 3, 5 or 7 days after challenge with *Bremia lactucae* isolate CL9W (Bremia), or inoculated with water alone (water). A, *LTC1*; B, *LTC2* and C, *triosephosphate isomerase* as a constitutively expressed control. Lanes show PCR products separated on 1% agarose gels. Amplification products were: A 462 bp; B 454 bp and C 741 bp.

Bremia lactucae (Figs. 6 and 7). Lettuce cv. Diana is resistant to the CL9W isolate of the downy mildew pathogen used and accumulates SL phytoalexins after challenge. Although RT-PCR analysis does not provide a truly quantitative measure of expression, the observed induction of *LTC1* and *LTC2* RNAs detected paralleled the reported accumulation of the phytoalexin lettucenin A in cotyledons after fungal infection (Bennett et al., 1994).

2.4. Single copies of LTC1 and LTC2 detected in lettuce

Probing Southern blots of lettuce DNA digested with *Bam*H1, *Eco*R1, *Hin*dIII, *Sst*I, *Xho*I and *Xba*I revealed up to three bands hybridising to *LTC2* at high stringency (data not shown). The hybridisation patterns were consistent with the presence of single copies of the sesquiterpene synthase genes in lettuce. We conclude that they are not part of a large gene family.

3. Concluding remarks

The two germacrene A synthases cloned from lettuce are strongly expressed in tissues which accumulate the guaianolide phytoalexin lettucenin A (Bennett et al., 1994; Bestwick et al., 1995). Our failure to isolate a sesquiterpene synthase cDNA with guaiene synthase activity indicates that biosynthesis of the induced SLs follows the route: FPP→germacrene A→germacranolide→guaianolide, as previously proposed for other species (Song et al., 1995; de Kraker et al., 1998, 2001). Seedlings with the red spot disorder were chosen for synthesis of the cDNA library because they accumulate not only phytoalexins, but also constitutive SLs within their developing laticifers. It is possible that the levels of constitutive expression of *LTC1* and *LTC2* detected in roots and hypocotyls provide sufficient enzyme activity for synthesis of the constitutive SLs, again via the germacranolide route, which are then distributed thoughout the seedling tissues. Alternatively, additional sesquiterpene synthases might provide the enzymes involved in the accumulation of SLs in laticifers.

Tobacco 5-*epi*-aristolochene synthase (TEAS) (Starks et al., 1997) produces its bicyclic product via germacrene A, that is not released from the active site. Comparison of the predicted active sites of LTC1 and LTC2 with that of TEAS, for which the crystal structure is known (Starks et al., 1997), using the SWISS-MODEL programme (www.expasy.org/sprot) revealed strong similiarities (see also Fig. 2) . The identification of *LTC1* and *LTC2* as germacrene A synthases should allow further assessment of the proposed mechanism of FPP ionisation and cyclisation by TEAS, particularly in relation to the generation of bicylic products.

In lettuce, the SLs represent good targets for genetic manipulation to achieve an enhanced phytoalexin response for disease resistance, and also changes in the profile of constitutive, latex-associated SLs to reduce bitterness or enhance resistance to insect pests. The germacrene A synthases *LTC1* and *LTC2* provide immediate targets for modified expression via sense and antisense transgenics in order to determine their function in plants, to further unravel the mechanisms of biogenesis of the biologically active SLs in lettuce and related species, and to generate beneficial modifications of the SL profile.

4. Experimental

4.1. Plants and fungi

Lettuce cv. Diana was used thoughout (seeds from Tozer Seeds, Cobham). The red spot disorder developed in seed stored for more than 1 year at 4 °C (Bestwick et al., 1995). Seedlings were grown and cotyledons inoculated with the downy mildew fungus (*Bremia lactucae*) as previously described (Bennett et al., 1994). For the developmental analysis of gene expression, seeds were surfaced sterilised with 15% v/v 'Domestos' bleach (15 min), washed thee times with sterile distilled water and plated out on Murashige and Skoog media (Duchefa) at pH 5.75 containing 0.3% phytogel, 2% sucrose w/v and 1x Gamborg's vitamins (Sigma). Seeds were germinated and grown at 25 °C in an illuminated chamber with a 16 h photoperiod. After 7 days seedlings were dissected into roots, hypocotyls, cotyledons and the first true leaves.

4.2. General molecular techniques

Total mRNA and DNA were isolated using the LiCl precipitation and phenol extraction methods, respectively, as described in Ausubel et al. (1999). PolyA

RNA, was isolated using Dynabeads (Dynal) and cDNA prepared using a Pharmacia cDNA synthesis kit according to the manufacturers instructions. The cDNA library was prepared in λGT11 using the protocol provided by our supplier, Promega. PCR was carried out with a Perkin Elmer 2400 thermal cycler using Taq polymerase from Bioline. For RT-PCR analysis of transcripts, total RNA (2 μg) was processed using the Qiagen Omniscript RT-PCR kit. Automated DNA sequencing utilised the ABI 310 system on products of a cycle sequencing kit.

Digestions with restriction enzymes (various sources), agarose gel electrophoresis of nucleic acids, Southern blotting and hybribisation with ^{32}P labelled probes (generated with a Pharmacia oligolabelling kit), plaque lifts and screening, and SDS/PAGE were carried out as described in Ausubel et al. (1999).

4.3. Primer pairs for PCR amplification of cyclases

Degenerate primers were forward: 5′ GCYRAYTT YYMYCCIISTITITGGGG and reverse: 5′ YYST-TYRTTWAYRTCYTTCCAT, later found to correspond to the peptide sequences RPLANFPPSVWG and WKDINEGCLKP respectively, in lettuce. For RT-PCR analysis of transcripts the gene-specific primers were *LTC1*, forward 5′GTTGAAGCCAATGGTACCC TT, reverse 5′CTTCACATCGTTGGTAATGGA; *LT C2*, forward 5′ GTTGACACTAATGCCACCATC, reverse 5′ CTTCACATCCGTAGAAATATC. The primers for *triosphosphate isomerase* cDNA which was used as a control were, forward 5′ GTTAAGAAG GGTGGTGCATT, reverse 5′ CTTATATCACCCATC TCATA. The programme used for RT-PCR was 5 min at 94 °C then 25 cycles of 10 s at 94 °C, 20 s at 54 °C and 60 s at 72 °C.

4.4. Bacteria, plasmid vectors and protein expression

E. coli strain DH12s was used as a host for routine cloning. Strain Y1090 was used as a host for phage λ, BMH 71–18muts (Promega) for mutagenesis, and BL21(DE3)pLysS (Promega) and BL21(DE3)RIL (Stratagene) were used for protein expression. Strains were grown using LB media (Ausubel et al., 1999), or LB supplemented with 1% glucose for expression experiments.

Site directed mutagenesis to remove an internal *Nco1* site was required prior to cloning into the expression vector pET28c(+) from Novagen. Mutagenesis was done as described by Deng and Nickoloff (1992). The predicted open reading frames for protein expression were amplified by PCR using the following primer pairs, *LTC1* and *LTC2* forward, 5′ TTCAAGCCATGGCA GCAGTTGA, *LTC1* reverse 5′GATCGTGCTCGAGT TATTACATAGGTGACG, *LTC2* reverse 5′GATCGT GCTCGAGTCATTACATGGATACAG. These primers engineered *Nco1* and *Xho1* sites at the 5′ and 3′ ends and enabled the expression of non-fusion protein.

4.5. Enzyme assays

The method used was based on that of Back et al. (1994). 50 ml of LB was inoculated from stock cultures and grown at 37 °C to an OD of 0.5 (600 nm), the culture was then induced with IPTG (1mM) and incubated for a further 16 h at 23 °C. The culture was centrifuged, the pellet washed and resuspended in 5 ml sesquiterpene assay buffer (SAB) comprising 20 mM MOPS, 10 mM NaCl, and 1% glycerol at pH7.0; containing $MgCl_2$ (10 mM) and DTT (1 mM). Lysozyme (1 mg/ml) was added and the cells incubated on ice for 30 min. Following sonication the lysate was centrifuged at 15,000 *g* for 20 min. SAB (+$MgCl_2$ 10 mM) was added to the supernatant to give a final volume of 20 ml. 23 μM [1-^{3}H] FDP (4.7 μC/μmol^{-1}) was added, the reaction overlaid with 2 ml pentane and incubated at 22 °C for 6 h. The reaction mixture was extracted with pentane (3×5 ml), the pentane fractions combined, dried over $MgSO_4$ then passed though a column (1 cm^3, silica gel 40–60:$MgSO_4$ 9:1). The eluate was then distilled at 60 °C and the residual sesquiterpene fraction analysed by radio TLC (Merck silica gel F254 plates) and GC-MS. TLC plates were developed in hexane:ethyl acetate (9:1) prior to analysis with a Bioscan system 200 imaging system. GC–MS was used to identify the major product, germacrene A.

pH optima and requirement for Mg^{2+} were explored using small scale assays. Buffers selected for pH ranges were; MES 5.6–6.6, MOPS 6.5–7.5, EPPS 7.4–9.1. Reactions 50 μl, containing culture lysate (0.5 μl), buffer (50 mM), 23 μmM [1–^{3}H] FDP (18.6 μC/μmol^{-1}), NaCl (10 mM), glycerol (1%), $MgCl_2$ (10 mM) and DTT (1 mM) were incubated at 25 °C for 10 min, stopped by the addition of 50 μl KOH/EDTA (0.4 M/0.2 M) and extracted with 500 μl pentane. Incorporation into the pentane fraction was recorded by scintillation counting. To determine if Mg^{2+} was required, 50 μl of extract from *E. coli* expressing *LTC2* was desalted into SAB (-Mg^{2+}) though a 1 cm^3 Sephadex G15 column, the eluate assayed with Biorad reagent and protein containing fractions pooled prior to assaying in the presence or absence of $MgCl_2$ (10 mM).

4.6. GC–MS analysis

GC–MS was carried out on Kratos MS80RFA instrument, using cool (20° C) on-column injection or hot (250 °C) injection onto a BPX5 (SGE) capillary column (0.32 i.d×25 m). The temperature programme was 45 °C for 1 min, then 5 °C per min to 210 °C which was held for 30 min. A straight-chain alkane standard mix was co-injected with the samples. Spectra and Kovat's retention indices were compared with the Wiley library and published data (Adams, 1995).

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