Plant Mol Biol (2008) 68:633–651
DOI 10.1007/s11103-008-9399-0

Transcriptome analysis approaches for the isolation of trichome-specific genes from the medicinal plant *Cistus creticus* subsp. *creticus*

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Received: 20 May 2008 / Accepted: 31 August 2008 / Published online: 26 September 2008

Abstract *Cistus creticus* subsp. *creticus* is a plant of intrinsic scientific interest due to the distinctive pharmaceutical properties of its resin. Labdane-type diterpenes, the main constituents of the resin, exhibit considerable antibacterial and cytotoxic activities. In this study chemical analysis of isolated trichomes from different developmental stages revealed that young leaves of 1–2 cm length displayed the highest content of labdane-type diterpenes (80 mg/g fresh weight) whereas trichomes from older leaves (2–3 or 3–4 cm) exhibited gradual decreased concentrations. A cDNA library was constructed enriched in transcripts from trichomes isolated from young leaves, which are characterized by high levels of labdane-type diterpenes. Functional annotation of 2,022 expressed sequence tags (ESTs) from the trichome cDNA library based on homology to *A. thaliana* genes suggested that 8% of the putative identified sequences were secondary metabolism-related and involved primarily in flavonoid and terpenoid biosynthesis. A significant proportion of the ESTs (38%) displayed no significant similarity to any other DNA deposited in databases, indicating a yet unknown function. Custom DNA microarrays constructed with 1,248 individual clones from the cDNA library facilitated transcriptome comparisons between trichomes and trichome-free tissues. In addition, gene expression studies in various *Cistus* tissues and organs for one of the genes highlighted as the most differentially expressed by the microarray experiments revealed a putative sesquiterpene synthase with a trichome-specific expression pattern. Full length cDNA isolation and heterologous expression in *E. coli* followed by biochemical analysis, led to the characterization of the produced protein as germacrene B synthase.

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Keywords *Cistus creticus* subsp. *creticus* · DNA microarrays · EST analysis · Gene expression · Germacrene B synthase · Trichomes

Abbreviations

ABA	Abscisic acid
aRNA	Amplified RNA
CcEF1α	*Cistus creticus* elongation factor 1α
CcGer	*Cistus creticus* germacrene B synthase
CER1	ECERIFERUM1 sterol desaturase
DTT	1,4-Dithiothreitol
EST	Expression sequence tag
FDP	Farnesyl diphosphate
GC/MS	Gas chromatography/mass spectrometry
GGDPS	Geranyl-geraniol diphosphate synthase
MIPS	Munich Information Center for Protein Sequences
NIST	National Institute of Standards and Technology
PDMS	Polydimethylsiloxane
SCID	Severe combined immunodeficiency disease
SEM	Scanning electron microscopy

Introduction

Cistus creticus subsp. *creticus* is a native member of the Mediterranean flora, widely known for the characteristic resin 'ladanon' excreted from leaves and stems, used since ancient times for its aromatic and pharmacological properties. Recent studies on plant extracts and isolated compounds from different *Cistus* species have shown strong gastric antiulcer activity (Attaguile et al. 1995; Yesilada et al. 1997a), antiproliferative and cytotoxic activity (Chinou et al. 1994; Dimas et al. 1998; Lendeckel et al. 2002), and antifungal, antibacterial, and anti-inflammatory properties (Chinou et al. 1994; Demetzos et al. 1997, 1999, 2001; Yesilada et al. 1997b). Main constituents of the aqueous extracts of *Cistus* species are flavonoids, whilst lipophilic extracts are enriched in labdane-type diterpenes (Demetzos et al. 1999). Among the compounds isolated and characterized are quercetin, flavan-3-ols, kaempferol and kaempferol-3-methyl ether, aesculin, myrecitin and various labdane-type diterpenes (Anastasaki et al. 1999; Chinou et al. 1994; Danne et al. 1994; Demetzos et al. 1989; Vogt and Gulz 1986). In particular, (13*E*)-labda-13-en-8α,15-diol and (13*E*)-labda-7,13-dienol, compounds belonging to the latter group, exhibited cytotoxic activity against human leukemic cell lines (Matsingou et al. 2005, 2006) and sclareol [(13*R*)-labda-14-en-8,13-diol] caused induced apoptosis in leukemia T-cell lines and in human breast cancer cells (Dimas et al. 2001, 2006). Recent studies indicated an improved antitumor activity of (13*E*)-labda-13-en-8α,15-diol and sclareol by the use of liposomal technology, as a drug carrier system, on human colon cancer tumors (HCT116) developed in SCID (Severe combined immunodeficiency disease) mice (Hatziantoniou et al. 2006).

The morphology of trichomes in the plant kingdom is highly diverse and it has been used as a taxonomic trait. Generally, trichomes are divided into two subcategories, glandular and non-glandular (Wagner et al. 2004). Commonly, glandular trichomes are the primary sites of secondary metabolite biosynthesis, as established by previous studies in other plant species. For example, the glands of *Mentha piperita* produce monoterpenes that contribute to the plant's characteristic odor (Lange et al. 2000). Furthermore, gland exudates of several Solanaceae species contain diverse compounds such as sesquiterpenes, acylsugars, methyl-ketons and diterpenes (Antonious 2001; Li et al. 1999). Sweet basil's phenylpropanoid production and storage takes place in the peltate glandular trichomes (Gang et al. 2001, 2002), while alfalfa possesses capitate glandular trichomes that produce and accumulate flavonoids and their derivatives (Aziz et al. 2005).

Both adaxial and abaxial surfaces of *Cistus creticus* subsp. *creticus* leaves are covered by multicellular glandular trichomes as well as non-glandular elongated and stellate trichomes. Glandular trichomes are approximately 100–250 μm long with a spherical head of 40–60 μm in diameter at the tip where the resin is excreted (Gulz et al. 1996). Histochemical studies have revealed polyphenol emmition of stellate trichomes in another *Cistus* species, *Cistus salvifolius*. These trichomes have a particularly dense arrangement at the border of crypts where stomata are mostly concentrated on the abaxial surface of the leaves (Tattini et al. 2007).

Essential oils produced by different plant species have been found in many cases to have pharmacological activity (Angelopoulou et al. 2001; Demetzos et al. 1997; Economakis et al. 1999). However, the metabolic pathways leading to the biosynthesis of many highly interesting compounds are often poorly characterized mainly due to limited genomic information from medicinal and aromatic plants. Functional genomic approaches have been applied successfully to identify genes coding for enzymes involved in secondary metabolism (Aziz et al. 2005; Covello et al. 2007; Shukla et al. 2006). Furthermore, cDNA libraries have been constructed and analyzed from glandular trichomes of *Artemisia annua* (Teoh et al. 2006), alfalfa (Aziz et al. 2005), mint (Lange et al. 2000), sweet basil (Gang et al. 2001), and wild and cultivated tomatoes (Fridman et al. 2005).

The production of plant secondary metabolites by the implementation of biotechnological approaches is critical in the case of low-level produced compounds or

compounds synthesized by rare, exotic or non-cultivated species. To achieve this goal of heterologous production, the isolation and characterization of trichome-specific genes and their promoters have been pursued (Teoh et al. 2006; Wang et al. 2002). In this study, transcriptome analysis of *Cistus creticus* glandular trichomes has been employed to gain insight into the biochemical pathways operating in this highly specialized tissue and to isolate and characterize trichome-specific genes.

Materials and methods

Plant material

Cistus creticus subsp. *creticus* plants used in this work were grown in pots containing a 3:1 mixture of perlite: soil: (a) in a Percival Scientific growth chamber under controlled environmental conditions (16 h day, 23°C and 8 h night, 18°C) and (b) in the greenhouse in the School of Agriculture, Aristotle University of Thessaloniki, Greece, under controlled temperature (25/18°C, day/night) and natural photoperiod.

Trichome isolation and RNA extraction

Trichomes were physically removed from leaves with the isolation procedure described previously (Yerger et al. 1992). Total RNA was extracted from the trichomes with the protocol developed previously for *Cistus creticus* tissues (Pateraki and Kanellis 2004) and DNA contamination was removed with RQ1 DNAse (Promega, Madison, WI, USA).

Metabolite profile analysis

Glandular and non glandular trichomes isolated from leaves at different developmental stages: stage 1 (~0.5 cm), stage 2 (1–2 cm), stage 3 (2–3 cm) and stage 4 (3–4 cm) were extracted in *n*-hexane for 18 h. Extracts were evaporated to dryness by a rotary vacuum evaporator at 40°C and subjected to gas chromatography/mass spectrometry (GC/MS) analysis. A HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) was used. The column was temperature programmed as follows: 110°C for 4 min, temperature increased to 230°C at a rate of 3.3°C/min and up to 250°C at a rate of 10°C/min (splitless mode). Mass spectrometry conditions were as follows: temperature of ion source 230°C, ionization energy 70 eV and electron current 1,453 μA.

cDNA library construction, sequence analysis and annotation

The cDNA library was constructed with the Creator Smart cDNA library construction kit (Clontech, Palo Alto, CA, USA) using 2 μg of total RNA as starting material. The recombinant phage DNA was packed with Gigapack III Gold Packaging Extract kit (Stratagene, La Jolla, CA, USA) as described in the instruction manual. Mass excision of the phage library into pTriplex2 resulted in recombinant plasmids isolated from *E. coli* cells by standard alkaline lysis protocol. Insert sizes were evaluated by restriction digest with *Hin*dIII and *Eco*RI endonucleases and subsequent electrophoretic analysis on agarose gel. Single pass, 5′ sequencing was performed for inserts larger than 400 bp by Lark Technologies Company. Raw sequence data were processed—vector sequences were trimmed—and high quality sequences were assembled by CAP3 algorithm (Huang and Madan 1999) into contigs. All trichome ESTs were analyzed by Blastx comparison to non-redundant (nr) protein database of GenBank. Functional annotation was achieved according to Munich Information Center for Protein Sequences (MIPS) Functional Catalogue Database (http://mips.gsf.de/).

Microarray production

A total of 1,248 cDNAs from 13 96-well plates were PCR-amplified, using Taq polymerase (Minotech, Heraklion, Greece) and common primers adjacent to the vector cloning sites (pTRIPLEX2F: 5′-GAA GCG CGC CAT TGT GTT GG-3′ and pTRIPLEX2R: 5′-GGC CGC ATG CAT AAG CTT GC-3′). The PCR products were assessed by agarose gel electrophoresis, purified using Nucleofast 96 columns (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol, and quantified. In addition, seven external control genes from *Bacillus subtilis* Dap (B3), Phe (B4), Lys (B5), *E. coli* BioB (C1), BioC (C2), BioD (C3) and phage P1 Cre (C4) were also amplified and included as positive controls and for grid-placement.

The purified PCR products (3 μg of each) were transferred in 384-well polypropylene printing plates (Genetix, Hampshire, UK), dried in room temperature and resuspended in printing buffer containing 450 mM NaCl, 45 mM phosphate pH 7.0, 5% [v/v] formamide and 0.01% [v/v] maltoside at a final concentration of 200 μg/μl. The printing, in triplicates, was performed on home-made aminosilane slides in the Post-Genomics Facility of IMBB-FORTH using a Packard SpotArray24 spotter with four print-tips.

RNA amplification/labeling

Amplification of 4 μg of RNA from stems and leaves without trichomes and roots from three individual plants as well as pools of RNA from trichomes was processed. The one-round amplification was performed according to a previously developed protocol (Van Gelder et al. 1990) with the following modifications: first strand synthesis was performed using an anchored T7-oligo(dT) primer and Superscript III (Invitrogen, Carlsbad, CA, USA) for 20 min at 44°C and 1 h 45 min at 50°C. Amplified RNA (aRNA) in vitro transcription was done with Ampliscribe T7 transcription kit (Epicentre, Madison, WI, USA) at 42°C following the manufacturer's protocol, using a ratio of UTP/aminoallyl-UTP (Epicentre, Madison, WI, USA) of 1:1. The aRNA was purified using RNeasy columns (Qiagen, Valencia, CA, USA) and quantified. Equal quantities of stem, root and leaf aRNA samples were labeled with Alexa 555 and 647 Succinimidyl ester (Molecular Probes, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol at 50°C. After column purification, the labeled aRNA was mixed with labeled mix of aRNA from the external control genes. B5, C1, C2 and C3 were labeled with Alexa 555, while C4, B3, B4 and B5 were labeled with Alexa 647.

Hybridization and microarray analysis

A total of 3 μg of labeled aRNA from the non-trichome mix labeled with one dye was co-hybridized with equal amount of the reciprocally-labeled aRNA from trichomes in a buffer with a final concentration of 5× SSC, 0.1% [v/v] SDS, 50% [v/v] formamide and 1.5 μg/ml fragmented salmon sperm DNA. Three biological replicate hybridizations with Alexa 647-labeled trichomes and one technical replicate (dye-swap) hybridization were performed in a Tecan HS4800 hybridization station. The slides were pre-hybridized for 1.5 h at 42°C with 5× SSC, 0.1% [v/v] SDS, 1% [v/v] BSA and then hybridized for 16 h at 42°C. The arrays were subsequently washed with 2× SSC, 0.1% [v/v] SDS at 42°C, followed by a wash in 0.1× SSC, 0.1% [v/v] SDS at 23°C and a third wash with 0.1× SSC at 23°C, and dried under nitrogen flow.

Arrays were read with a ScanArray 5000 scanner (GSI Lumonics, Wilmington, MA, USA) at 5 μm resolution at three different photomultiplier tube voltage settings (high, medium and low). The fluorescence intensity for each fluor and each element on the array was captured using ImaGene 5.0 (Biodiscovery, Marina del Rey, CA, USA). A single intensity value was computed from the three different settings as described previously (Dudley et al. 2002) with the following modifications: the linear regression was performed on the $\log_2$-transformed spot values. If more than one setting had values in the linear range of the scanner then the average of the intensities was computed. Weighted print-tip loess normalization was performed using package limma version "2.10.0" (Smyth et al. 2005) in R version 2.5.0 (Team 2007).

As the *Cistus* features on the array come from the trichome cDNA library, to ensure a more accurate fit of the loess curves, the buffer and housekeeping genes elements were given a weight of 4, while the external control genes were given zero weight. To identify the trichome-overexpressed genes, the differences in gene expression between the trichome and the non-trichome samples were computed by fitting a linear model on the *Cistus* genes (i.e. excluding buffer and external control features) using limma (Smyth 2004). The intra-array technical replication was estimated as described by Smyth et al. (2005). In order to treat the three biological replicates equally, the two dye-swapped hybridizations were given half weight. Genes having differential expression statistical significance smaller than 5% FDR-adjusted *P*-value (Benjamini and Hochberg 1995) and a change more than 2-fold were called differentially expressed.

RT-PCR analysis

Expression analysis of selected putative trichome-specific genes was performed by RT-PCR. Total RNA was isolated as described above and was used for first strand synthesis using the M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. PCR amplification was performed with *Taq* DNA polymerase (HyTest, Turku, Finland) with an initial denaturation step for 2 min at 94°C, followed by denaturation for 30 s at 94°C, annealing for 30 s at 68°C, extension for 1 min at 72°C and a final extension of 5 min for a defined number of cycles for each gene in order to avoid transcript saturation conditions. *Cistus creticus* elongation factor 1α (*CcEF1α*, accession number EF062868) transcript levels were used as internal control. The primer sequences used were: 3150F (5′-CGT TGA TCG CCT TCT TCT TC-3′), 3150R (5′-CGT CCG AGA GAC TCA CAT CA-3′), 1178F (5′-ATT TCT TCC CCA ATG GAA CC-3′), 1178R (5′-AAC CAA AAT CAT GCC TCC AC-3′), 3096F (5′-TGG GGA TTT TGC AGA TTC AT-3′), 3096R (5′-GCC CTA GAC AAA AGG ACT GC-3′), 663F (5′-TCA TCA CCT GCA CTT TGC TC-3′), 663R (5′-GAC CTG GTG GAA ATG GAG AA-3′), 3012F (5′-GGT TGT TGG GTT CTT GGC TA-3′), 3012R (5′-CAT CTT AAC TAC GCA TTG CAC A-3′), 1756F (5′-TGG AGC ACA ACT CAA AGC TG-3′), 1756R (5′-CCA ACT TCC CAG AAA ACC AA-3′), 2655F (5′-AGG CAT GCA CAT CCA CCT-3′), 2655R (5′-GGG CAA TCG AAA CGT CTA AG-3′), 2832F (5′-AAT GGA GGC ACA

AAT GGA AG-3′), 2832R (5′-TAA CCG GAG CTA ACC AAT GC-3′), EF1αF (5′-GGT CCT ACT GGT TTA ACC ACT G-3′) and EF1αR (5′-CTC GGA GAA GGT CTC CAC AAC C-3′).

Isolation and heterologous expression of *Cistus* germacrene B synthase

5′ cDNA end amplification of *Cistus* germacrene B synthase (*CcGer*) was accomplished by PCR on phage DNA as template. The primers used were a gene-specific reverse primer designed on the sequence provided by the EST analysis for the gene of interest (2832R: 5′-GTG TAC TTT GGC TCA AAA TAC ACT CC-3′) and a forward primer designed on pTriplex2 vector sequence upstream the cloning site. (pTriplex2F: 5′-CTC CGA GAT CTG CAC GAG C-3′). The full length cDNA was obtained by PCR and phage DNA as template using the Pfx50 proofreading polymerase (Invitrogen, Carlsbad, CA, USA). The primers used were: FL2832F: 5′-GCT AGC ATG TTT ACC CAA ACA TCA TTA TCA TC-3′ (containing a *Nhe*I site) and FL2832R: 5′-GAG CTC CAC TTT CAT GGG ACC GGA TTG ATG AGC-3′ (containing a *Sac*I site). The resulting fragment was cloned into the vector pET21A (Novagen, Madison, WI, USA). *E. coli* BL21 Codon Plus competent cells were transformed and used for protein expression. Induction was performed in a 50-ml liquid culture incubated overnight at 18°C with 400 μM isopropyl-1-thio-*β*-galactopyranoside.

Enzyme assay

Cells from the induced culture were harvested by centrifugation (2,000*g*, 15 min) at 4°C and resuspended in 5 ml of extraction buffer 50 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid, pH 7.0, 10% [v/v] glycerol, 5 mM $MgCl_2$, 5 mM 1,4 dithiothreitol (DTT), 5 mM sodium ascorbate, and 0.5 mM phenylmethylsulfonyl fluoride. Disruption of the cells was performed by sonication (Bandelin UW2070). Homogenates were then centrifuged at 20,000*g* at 4°C for 30 min. The supernatant was desalted with a Bio-Rad Econo column and 5 ml of assay buffer, containing 10 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid, pH 7.0, 10% [v/v] glycerol, and 1 mM DTT was used in the final elution step. The enzyme reaction contained 960 μl of crude protein extract, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 mM $NaWO_4$, 0.1 mM NaF, and 60 μM farnesyl diphosphate (Echelon Biosciences, Salt Lake City, UT, USA). Assay mixtures were incubated at 30°C for 1 h. A solid-phase microextraction PDMS-100 (polydimethylsiloxane) fiber (Supelco, Bellefonte, PA, USA) was inserted into the tube containing the reaction for 15 min at 42°C for collection of the volatiles. The solid-phase micro-extraction fiber was then injected into a gas chromatography–mass spectrometry system for analysis. Negative control included the reaction with crude extracts of induced *E. coli* carrying the expression vector with different insert and 60 μM of farnesyl diphosphate.

Gas chromatography/mass spectrometry analysis

Gas chromatography/mass spectrometry (GC/MS) analysis was conducted using a Shimadzu QP-5000 system (Shimadzu Corporation, Kyoto, Japan) fitted with an Alltech EC-5 column (0.32 mm in diameter, 30 m long, 1 μm film thickness) and equipped with Shimadzu GC-17 gas chromatographer. Samples were injected onto the column at 250°C in the splitless mode. After a 2 min isothermal hold at 50°C, the column temperature was increased by 10°C/min to 275°C with a 10 min isothermal hold at 275°C. The flow rate of the helium carrier gas was 1.4 ml/min.

Scanning electron microscopy (SEM)

Pieces of young (approximately 1 cm) and fully-expanded (approximately 3–4 cm) leaves of *Cistus creticus* were initially fixed for 3 h with 6% [v/v] glutaraldehyde in 0.024 M phosphate buffer, pH 6.8. After several washes in buffer, the specimens were postfixed with 1% [v/v] osmium tetroxide similarly buffered (4°C, 5 h). Tissue dehydration was carried out in an acetone series followed by critical point drying (Balzers CPD 030) and carbon coating (vacuum evaporator JEE-4X). Observations were made on a JEOL JSM-840A scanning electron microscope operating at 15 KV.

Results

C. creticus trichomes' structure and chemical composition

Leaves of *C. creticus* bear on their surface two types of trichomes, glandular and non-glandular ones (Fig. 1a–d). Glandular trichomes are elongated and consist of a group of cells, from which those being close to the base are flattened, whereas the ones approaching the tip become fine cylindrical (Fig. 1b). The diameter of the trichome at the base is about 25 μm and at the tip 5 μm. The apical region ends in a small globular/ovoid head (Fig. 1a, b). Non-glandular trichomes are of two types, i.e. stellate multicellular trichomes composed of unicellular rays (Fig. 1b, e) and simple long trichomes with acute tips (Fig. 1c). In young leaves, the glandular and non-glandular pubescence is very thick (Fig. 1d), whereas later at full leaf expansion, trichomes become remarkably thinned (Fig. 1e). The

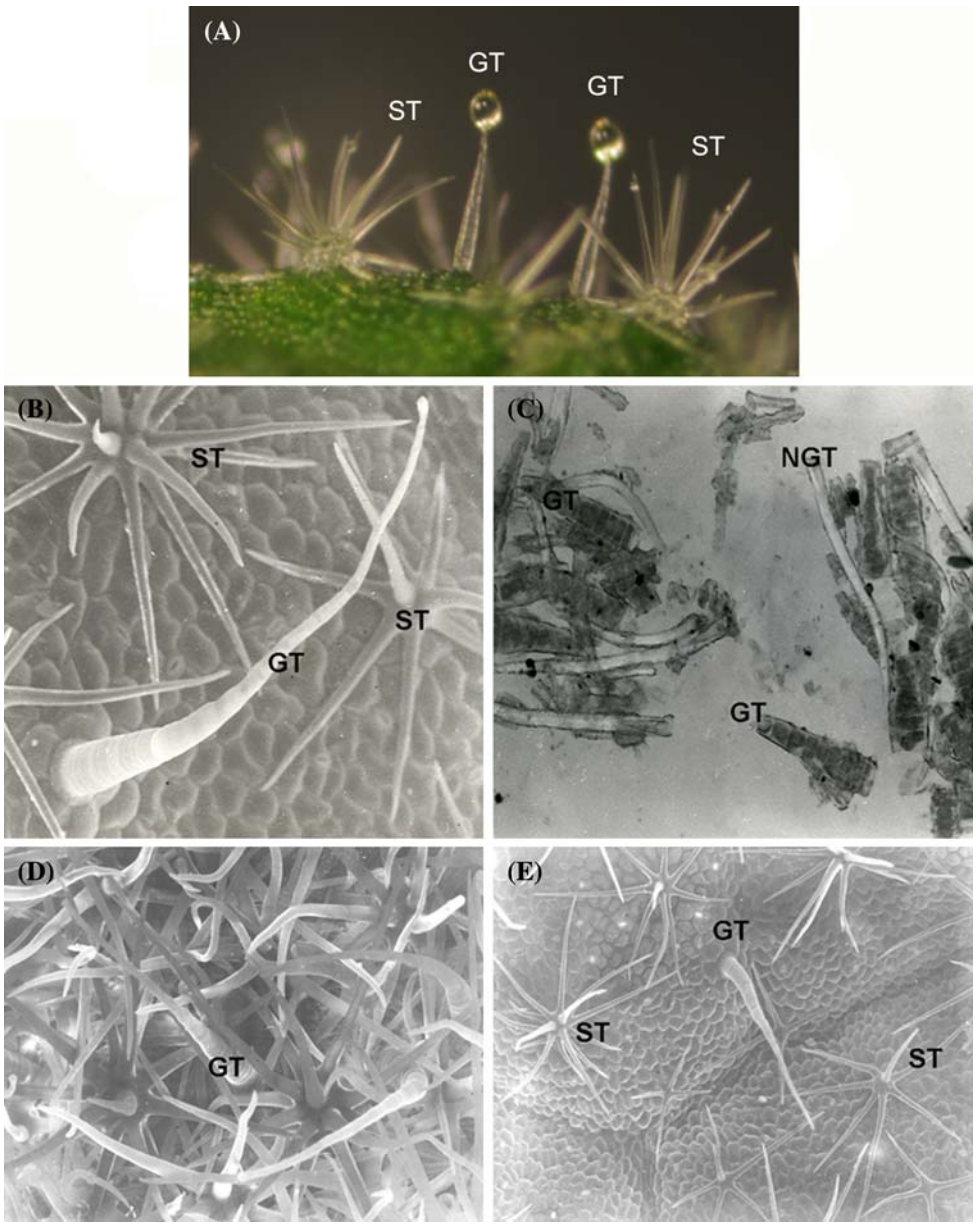

Fig. 1 Trichome types on leaf surface of *Cistus creticus*. (**a**) Stereoscope view of glandular trichomes (GT) and stellate trichomes (ST). (**b**) SEM view of glandular (GT) and stellate trichomes (ST) (×300). (**c**) Sample preparation of isolated trichomes (NGT: non glandular simple trichome). (**d**, **e**) Surface view by SEM of young (**d**, ×220) and fully expanded leaf (**e**, ×110)

isolation of trichomes according to the protocol described by Yerger et al. (1992) resulted in a sample preparation of both glandular and non-glandular trichomes (Fig. 1c).

The key step for successful application of EST analysis for the identification of genes participating in secondary metabolite biosynthesis is the use of appropriate tissue (and subsequently mRNA) characterized by high biosynthetic rates e.g. appropriate leaf developmental stage (Lange et al. 2000). To identify this stage, total trichomes were isolated and chemically analyzed from four developmental stages of *C. creticus* leaves: stage 1 (0.5–1 cm), stage 2 (1–2 cm), stage 3 (2–3 cm) and stage 4 (3–4 cm) (Fig. 2). Several peaks, mainly corresponding to labdane-type diterpenes, other diterpenes and sesquiterpenes, were determined after extraction of lipophilic compounds with hexane and GC/MS analysis. The detected compounds were identified with comparison of their mass spectra to those of authentic samples, using retention indices and bibliographic data (Anastasaki et al. 1999; Demetzos et al. 1999). Qualitative analysis of the constituents showed the existence of sesquiterpenes and diterpenes with predominant the labdane-type diterpenes (Fig. 2). Trichomes isolated from stage 2 displayed the highest content of labdane-type diterpenes (80 mg/g fresh weight) whereas trichomes from stages 3 and 4 exhibited gradual decreased concentrations. On the other hand, it was interesting to note that sesquiterpene levels showed an opposite trend by increasing in later stages i.e. in older leaves. Other diterpenes, such as clerodanes and kaurenes, were absent in the last three developmental stages. This trend was further

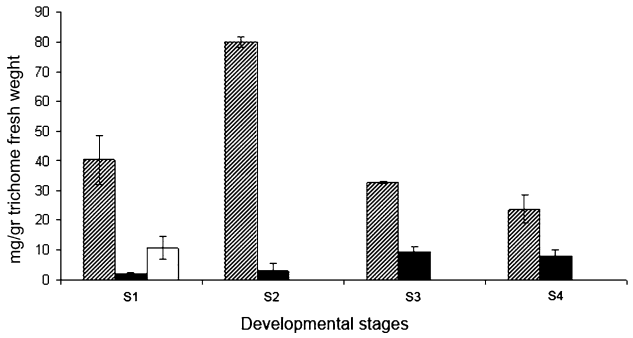

Fig. 2 Terpenoid content of *Cistus creticus* ssp. *creticus* trichomes at different leaf developmental stages. Accumulation of each class of terpenoids (mg/g fresh wt). Hatched bars represent labdane-type diterpenes, black bars sesquiterpenes and open bars other diterpenes. Leaf developmental stages: S1, 0.5–1 cm; S2, 1–2 cm; S3, 2–3 cm; S4, 3–4 cm. Error bars represent SEs

verified by RT-PCR analysis of *C. creticus* geranylgeranyl diphosphate synthase genes at identical developmental stages (Pateraki and Kanellis 2008), indicating that diterpene production is closely correlated with the expression of this gene coding for the first enzyme in the terpenoid pathway dedicated in diterpene biosynthesis.

EST analysis of *C. creticus* trichome cDNA library

Based on the above findings and in order to assure that the *Cistus* trichome cDNA library would be rich in transcripts participating in diterpene and sesquiterpene biosynthesis, it was deemed necessary to isolate trichomes and subsequently total RNA from three developmental stages, namely S1, S2 and S3, that showed the highest terpene accumulation (Fig. 2). Therefore, a cDNA library was constructed by mixing total RNA from these three stages at a ratio of 1:2:1. The primary library was characterized by a titer of approximately 1×10^6 pfu/ml and 92% recombinant clones. Initially, 2,743 library clones were randomly selected for single pass sequencing and subsequent vector sequence trimming resulted in 2,022 clones of high-quality sequence. These ESTs were in silico analyzed using Blastx algorithm to non-redundant (nr) protein database. Furthermore, sequences were clustered by contig assembly software CAP3 into 296 contigs and 1,071 singletons. Thus, the redundancy of the EST collection was calculated to be 47% (number of ESTs in clusters/total number of ESTs). Moreover, low redundancy was justified by most of the clusters being rather small with 258 out of 296 consisting of 2–4 ESTs and only eight consisting of more than 10 ESTs (Table 1).

Functional annotation was consigned to all 1,367 unique ESTs based on similarity to *Arabidopsis thaliana* annotated genes according to MIPS FunCatDatabase (http://mips.gsf.de/) (Ruepp et al. 2004) (Fig. 3). The largest group of

Table 1 Trichome cDNA library redundancy level

No of ESTs/contig	No of contigs
2–4	258
5–10	35
11–20	5
21–50	3

ESTs (38%) comprised of sequences coding for peptides of unknown function, either because of lack of homology to deposited sequences to public databases or homology to non-annotated homologous genes. Transcripts of housekeeping genes coding for ribosomal and photosynthesis-related proteins belonged to the second and third largest group, represented, in both cases, by 13% of total transcripts. The next category corresponded to secondary metabolism-related genes comprising 5% of total ESTs (8% of annotated ESTs) implicated in the principal function of glandular trichomes. The next largest group included stress response-related ESTs (4%) followed by functional groups involved in lipid, fatty acid and isoprenoid metabolism (3%), energy (3%), protein degradation (3%), C-compound and carbohydrate metabolism (3%) and protein folding and stabilization (2%) (Fig. 3).

The 10 most abundant transcripts in the library were determined after elimination of ribosomal and photosynthesis related genes, to enhance comprehension of the principal role and function of *C. creticus* trichomes (Table 2). An ubiquitin-like protein represented by the greatest number of ESTs was found on top of this record. An equivalent number of ESTs were identified for a sterol desaturase and a unigene with no similarity to known sequences. Lipid transfer proteins are of high abundance in

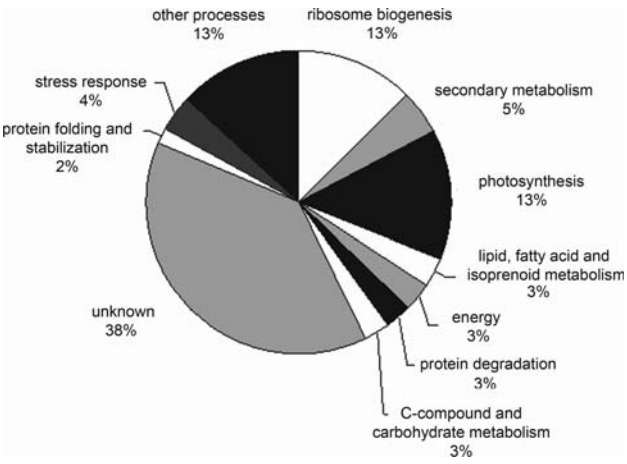

Fig. 3 EST analysis of *Cistus creticus* trichome cDNA library. Functional classification of 2,022 transcripts according to MIPS Functional Catalogue Database (http://mips.gsf.de/). Gene classes are indicated in the graph with different color

Table 2 Most abundant ESTs from *Cistus creticus* subsp. *creticus* trichome library with gene annotation of their closest hit identified by Blastx and the corresponding *E*-value

No of ESTs/contig	Homology (Blastx to nr database)	*E*-value
21	Ubiquitin-like protein (*Pisum sativum*)	4e-42
11	Sterol desaturase (*Medicago truncatula*)	2e-55
11	No significant similarity	
8	Fiber lipid transfer protein (*Gossypium barbadense*)	7e-34
8	F1F0-ATPase inhibitor protein (*Arabidopsis thaliana*)	5e-26
7	Ultraviolet-B-repressible protein (*Gossypium hirsutum*)	2e-15
7	ABA stress ripening protein (*Mesembryanthemum crystallinum*)	4e-14
6	Heat shock 20 protein-like (*Arabidopsis thaliana*)	2e-39
6	Aldehyde dehydrogenase (*Medicago truncatula*)	3e-46
6	No significant similarity	

the glands of several species (Fridman et al. 2005; Gang et al. 2001) and one of them appeared to be vastly transcribed in *C. creticus* trichomes. Additionally, among the 10 most abundantly transcribed genes, we identified cDNAs coding for a F1F0-ATPase inhibitor protein, an ultraviolet-B-repressible protein, an absisic acid (ABA) stress ripening protein, a heat shock protein, a cytosolic aldehyde dehydrogenase and one additional protein of unknown function.

Secondary metabolism-related genes, components of the fourth largest EST category, were classified into four groups: terpenoid, flavonoid, alkaloid and phenylpropanoid biosynthesis related genes (Table 3). Approximately 20% of the secondary metabolism-related cDNAs are associated with the terpenoid biosynthetic pathways (Fig. 4). This cluster is comprised of four unigenes (derived from six ESTs) with high sequence similarity to sesquiterpene synthase genes, two identical ESTs showing significant homology to diterpene synthase genes, one EST coding for triterpenoid cyclase and two unigenes corresponding to 1-deoxy-D-xylose 5 phosphate synthase (2 ESTs) and 3-hydroxy-3-methylglutaryl-CoA reductase (2 ESTs). These last two enzymes function in the plastidial and mevalonic pathways, respectively. Additionally, there are 2 ESTs related to monoterpene biosynthesis with similarities to a 10-hydroxygeraniol oxidoreductase and a putative acetyl-CoA geraniol/citrinellol acetyltransferase gene. Phenylpropanoid biosynthetic enzymes in *C. creticus* trichomes are represented by ESTs corresponding to several genes of the pathway such as cinammate 4-hydroxylase (2 ESTs), cinnamyl alcohol dehydrogenase (4 ESTs), cinnamoyl CoA reductase (1 EST), caffeic acid *O*-methyltransferase (4 ESTs) and eugenol *O*-methyltransferase (1 EST). Flavonoid biosynthesis-associated genes include four ESTs coding for chalcone synthase (2 different unigenes), two for flavonol synthases, one for 2′-hydroxy isoflavone/dihydroflavonol reductases, six for isoflavone reductases (3 unigenes) and one for anthocyanidine reductase. Three of the cDNAs encode enzymes probably participating in alkaloid biosynthesis and share similarities with a putative tropinone reductase, a putative salutaridinol 7-*O*-acetyltransferase and a putative desacetoxyvindoline 4-hydroxylase. Furthermore, five ESTs are similar to cytochrome P450 genes, two of which are related to secondary metabolism, one to lipid metabolism and one to a gene of unknown function, based on their homology to *Arabidopsis thaliana* P450 subfamilies. All the above transcripts reflect the production of highly diverse secondary metabolites in *C. creticus* trichomes.

Microarray analysis and trichome preferentially expressed genes

The EST analysis of the *C. creticus* trichome cDNA library led to the identification of 1,248 unique sequences that were used to construct a custom DNA microarray. Due to the source of the cDNA library there was a need for a robust alternative normalization method. Therefore the standard print-tip loess normalization was used, but spots corresponding to features expecting to have roughly equal intensities in both trichome and non-trichome samples were given a bigger weight (see Section "Materials and methods"). To evaluate not only the average fold-change, but also the significance of differential expression across the biological replicates a statistical cut-off of 5% FDR-adjusted *P*-value cut-off 30 was applied. Using both the statistical cut-off and a 2-fold increase in the trichome samples, the transcriptional comparative analysis of trichomes, stems and leaves without trichomes as well as roots revealed trichome preferential expression for 133 ESTs. Functional categorization of these ESTs is shown in Fig. 5. In the same plot percentage (%) of total library ESTs in the same functional classes is presented. It is noted that 23% of genes preferentially expressed in the trichomes were of unknown function and this class of sequences remains the most abundant. Secondary metabolism was the second most abundant class represented by 13% of these genes (compared to 5% of total genes). In addition energy, lipid

Table 3 ESTs coding for proteins with similarity to known enzymes of several secondary metabolite biosynthetic pathways in *Cistus creticus* trichome library. Accession number of the closest hit by Blastx analysis and *E*-value for the longest EST of each contig are provided

Homology to	Accession number of closest hit (Blastx)	*E*-value	No of ESTs/ contig
Terpenoid pathway			
3-Hydroxy-3-methylglutaryl-coenzyme A reductase	ABL10110	7e-75	2
1-Deoxy-D-xylulose 5-phosphate synthase	ABL10109	6e-70	2
Sesquiterpene synthase 1	AAR99061	8e-40	1
Sesquiterpene synthase 2	Q39761	1e-27	2
Sesquiterpene synthase 3	AAS66357	1e-15	1
Sesquiterpene synthase 4	AAS66358	9e-35	2
Diterpene synthase	O04408	0.005	2
2,3-Oxidosqualene-triterpenoid cyclase	AY104091	8.6	1
Geraniol dehydrogenase	AAX83107	2e-07	1
Acetyl CoA geraniol/citronellol acetyltransferase	AAW31948	1e-18	1
Alkaloid pathway			
Tropinone reductase	CAO02390	6e-07	1
Salutaridinol 7-*O*-acetyltransferase	Q94FT4	5e-22	1
Desacetoxyvindoline 4-hydroxylase	AAC49826	5e-30	1
Flavonoid pathway			
Chalcone synthase 1	AAG43353	4e-11	2
Chalcone synthase 2	Q9FUB7	2e-121	2
Flavonol synthase/flavanone 3-hydroxylase	Q9ZWQ9	1e-82	2
2′-Hydroxy isoflavone/dihydroflavonol reductase	AF202182	4e-28	1
Isoflavone reductase 1	AF071477	e-134	4
Isoflavone reductase 2	NM_121871	3e-88	1
Isoflavone reductase 3	AF282850	3e-35	1
Anthocyanidin reductase	ABM64802	5e-67	1
Phenylpropanoid and lignin pathway			
Cinammate 4-hydroxylase	CK085596	0.26	2
Cinnamyl alcohol dehydrogenase	AF320110	8e-66	4
Cinnamoyl CoA reductase	AAP46143	1e-117	1
Caffeic acid *O*-methyltransferase	O23760	2e-35	4
Eugenol *O*-methyltransferase	AB086103	7e-30	1

metabolism, transport facilitation and cell wall-related genes appeared to have stronger differential expression in trichomes indicative of a high metabolic activity.

To further confirm the existence of trichome-specific genes, we included in the microarray a set of external control genes. Recent work has relied extensively or exclusively on such controls to estimate the quality of the normalization, especially in cases of global mRNA changes (van de Peppel et al. 2003). In our experiments the corresponding RNA of the external controls was labeled only with one dye to serve as a positive control for tissue specificity of the corresponding labeled sample. By using this approach many technical limitations of the microarray technology, like channel bleeding and overall cross-hybridization, were overcome. This approach led to the identification of fifteen sequences as potential trichome-specific genes (Fig. 6). These genes encode two thaumatin-like proteins (2655 and 1756), a putative sesquiterpene synthase (2832), a putative short-chain alcohol dehydrogenase (3012), two cytochrome P450 monooxygenase-like proteins (663 and 3096), a cytosolic aldehyde dehydrogenase (383), a photosystem I subunit (1201), a putative 9-cis-epoxycarotenoid dioxygenase (651), an alpha expansin precursor (2019) and a protein of unknown function (3150) in addition to three ESTs with no significant similarity to known sequences (1220, 1178 and 1905). It is noteworthy that these cDNAs primarily correspond to secondary metabolism and defence-related proteins with higher expression levels in trichomes ranging from 15.37 to 1.05-fold compared to tissues without trichomes (Table 4). Six of them were selected for further verification by RT-PCR with gene-specific primers using total RNA extracted from various *C. creticus* tissues (Fig. 7a, b). The tissues under investigation were leaves, leaves without trichomes,

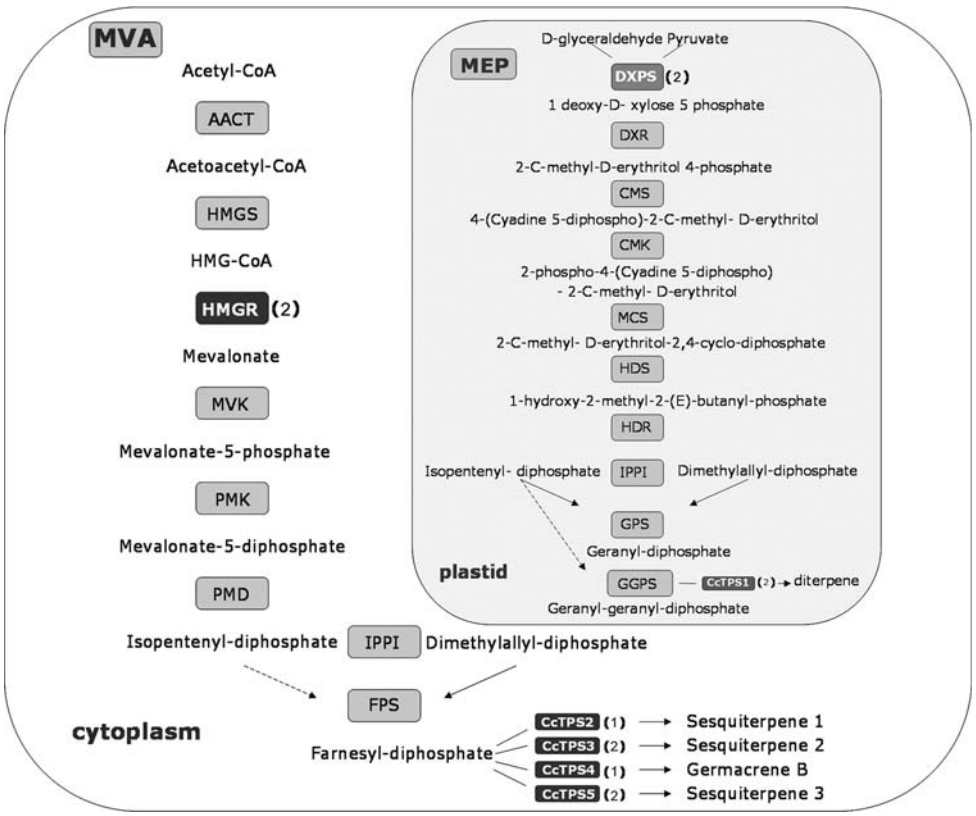

Fig. 4 Terpenoid biosynthesis in *Cistus creticus* trichomes: genes and metabolic intermediates. Mevalonic acid (MVA) pathway is localized in the cytosol and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is localized in the plastids. The enzymes participating in these pathways are indicated in boxes: AACT, acetoacetyl CoA thiolase; HMGS, hydroxymethylglutaryl coenzyme A synthase; HMGR, hydroxymethylglutaryl coenzyme A reductase; MVK, mevalonate kinase; PMK, mevalonate 5-phosphate kinase; PMD, 5-diphosphomevalonate decarboxylase; IPPI, isopentenyl diphosphate isomerase; FPS, farnesyl diphosphate synthase; DXPS, deoxyxylulose 5-phosphate synthase; DXR, deoxyxylulose 5-phosphate reductoisomerase; CMS, 4-diphosphocytidyl-methylerythritol synthase; CMK, 4-diphosphocytidyl-methylerythritol kinase; MCS, methylerythritol 2,4-cyclodiphosphate synthase; HDS, hydroxymethylbutenyl 4-diphosphate synthase; HDR, hydroxymethylbutenyl 4-diphosphate reductase; GPS, geranyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; CcTPS1-5, *Cistus creticus* terpene synthases. Dark boxes indicate enzymes followed by the number of ESTs found during the *Cistus creticus* trichome EST analysis

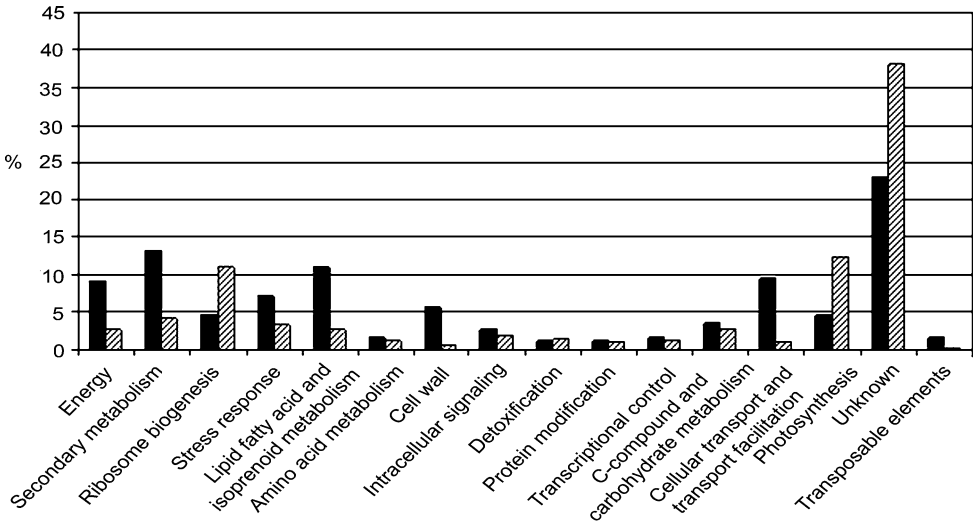

Fig. 5 Functional categorization of trichome preferentially expressed genes according to microarray transcriptome analysis between trichomes and trichome-free tissues. Percentage of differentially expressed genes (black bars) in each gene class is compared to that of total genes of the same functional class (hatched bars) represented in the cDNA library

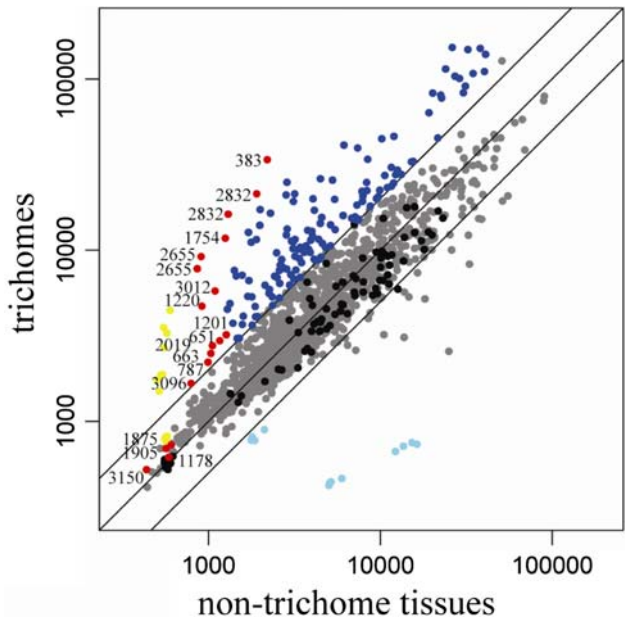

Fig. 6 Detection of genes with enhanced expression in *C. creticus* trichomes by differential microarray analysis. Scatterplot of 'normalized genes' fluorescence in trichomes versus non-trichome tissues, as measured after fitting the linear model. Yellow and cyan points represent the positive and negative external controls, respectively. Black points represent spots given additional weight in the normalization, including buffer spots and housekeeping genes. With blue the identified, using 2-fold increase and 5% FDR-adjusted *P*-value cutoffs, trichome preferentially expressed genes are depicted, while with red the potential trichome specific ones. Grey points represent *C. creticus* genes not belonging to any of the above categories. The black lines represent from left to right 2-fold, no change and 0.5-fold

stems, stems without trichomes, trichomes, roots, buds, petals, sepals, seeds and flower reproductive organs. Our results showed that EST 2832 with high similarity to sesquiterpene synthases was detected only in trichomes and not in leaves and stems without trichomes as well as in other tissues, i.e. roots, buds, petals, sepals, seeds. However, a minimum expression was noticed in flower reproductive organs (stamens, anthers, ovary, style and stigma) (Fig. 7a). This could be attributed to trichome-type structures present on these organs. ESTs corresponding to thaumatin-like protein (2655) and putative short-chain alcohol dehydrogenase (3012) (Fig 7a), as well as putative cytochrome P450 (3096) and protein of unknown function (1178) (Fig 7b) were expressed mainly in trichomes. Detection in other tissues tested i.e. buds, petals, sepals and reproductive organs could be also assigned to trichomes present on these tissues. Verification of trichome preferential expression failed in the case of 3150 (unknown function) exhibiting a relatively low fold change (1.2) of expression (Fig. 7b). The false estimation of expression specificity for this gene was probably due to its low expression levels; 41 cycles of amplification were essential for detectable transcripts.

Functional characterization of a trichome-specific germacrene B synthase

The trichome-specific cDNA 2832 (1,114 bp) showed significant similarity (71% at protein level) with *Populus balsamifera* subsp *trichocarpa* × *Populus deltoides* (−)-germacrene D synthase (GenBank accession number AAR99061). DNA from phage library was used as template to obtain the 5′ end of the cDNA and the subsequent isolation of the 1,727 bp full-length cDNA (*CcGer*, Accession number EU726270). The open reading frame (ORF) of the gene encodes a peptide of 572 amino acids with an estimated molecular mass of 65.8 kD and a p*I* of 5.51, similar to sesquiterpene synthases from other angiosperm species. CcGer shares 58% amino acid identity with *Populus balsamifera* subsp *trichocarpa* × *Populus deltoides* (−)-germacrene D synthase (GenBank accession number AAR99061), 51% identity with *Gossypium arboreum* (+)-delta-cadinene synthase (GenBank accession number CAA65289), 49% identity with *Citrus sinensis* valencene synthase (GenBank accession number AAQ04608) and 48% identity with *Cucumis sativus* β-caryophyllene synthase (GenBank accession number AAU05952).

A comparison of the protein sequence of *CcGer* with sesquiterpene synthases from *Vitis vinifera* (germacrene D synthase, Genbank accession number: AAS66357), *Citrus sinensis* (valencene synthase, Genbank accession number: X96429) and *Gossypium arboreum* (cadinene synthase, Genbank accession number: AAQ04608) shows that the deduced amino acid sequence of *CcGer* contains the characteristic sequence motifs of the TPS family including the active site DDXXD motif, involved in binding of the divalent metal ion (Mg^{+2}) required for its catalytic activity and the highly conserved RRX_8W motif (Fig. 8).

The catalytic capability of CcGer could not be deduced from its primary structure but the lack of an encoded N-terminal plastidial leading peptide suggested a sesquiterpene synthase. The ORF of *CcGer* was cloned into pET21A expression vector and the resulting plasmid was transformed into *E. coli* BL21 Codon Plus cells. Crude total protein extracts from *E. coli* cells harboring the *CcGer* were tested for sesquiterpene synthase activity. Indeed, CcGer was able to use farnesyl diphosphate (FDP) as substrate in vitro and convert it into one major product, germacrene B, as determined by GC/MS analysis of reaction products (Fig. 9). This was primarily supported by the observed significant similarities of the mass spectrum peaks of the compound with those of germacrene B deposited in National Institute of Standards and Technology (NIST) databases (Fig. 9c). Comparison of this compound with authentic germacrene B from *Lysopersicon hirsutum* extract provided by C. Sallaud (Van der Hoeven

Table 4 Potential trichome-specific genes

EST	Fold change trichome/ stem_root_leaf	Homology	Putative function
2655	10.12	Thaumatin-like protein	Defence related proteins
2832	12.47	(−)-germacrene D synthase	Secondary metabolism
1754	9.38	Thaumatin-like protein	Defence related proteins
1220	5.14	No significant similarity	Unknown
3012	5.29	Putative short-chain alcohol dehydrogenase	C-compound and carbohydrate metabolism
787	2.23	No significant similarity	Unknown
663	2.42	Cytochrome P450 monooxygenase like protein (CYP71B4)	Secondary metabolism
3096	2.11	CYP77B1; heme binding/iron ion binding/monooxygenase/ oxygen binding	Detoxification
3150	1.20	Translocon-associated protein beta	Unknown
1178	1.05	No significant similarity	Unknown
383	15.37	Cytosolic aldehyde dehydrogenase RF2C	Energy conversion and regeneration
1201	2.53	Photosystem I subunit	Photosynthesis
1905	1.23	No significant similarity	Unknown
651	2.55	9-cis-epoxycarotenoid dioxygenase, putative	Cell growth/morphogenesis
2019	2.63	Alpha-expansin precursor	Cell wall

Putative function is assigned to ESTs according to MIPS Fan Cat Catalogue Database

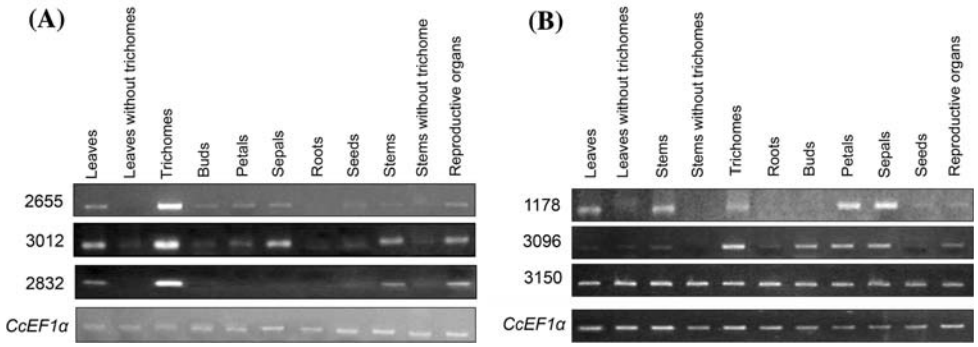

Fig. 7 RT-PCR analysis of transcript levels of putative trichome—specific genes in *Cistus creticus* tissues. The clones examined were: (**a**) 2832, germacrene B synthase; 3012, similar to short-chain alcohol dehydrogenase; 2655, similar to thaumatin and (**b**) 1178, with no significant similarity; 3096, similar to cytochrome P450; and 3150 similar to unknown protein. *Cistus creticus* elongation factor 1α (*CcEF1α*) was used as internal control

et al. 2000) yielded an identical retention time (Fig. 9a, b) and mass spectrum (Fig. 9c, d). A smaller portion of δ-elemene, which appeared as a minor peak in the chromatograph, is regularly considered as degradation product of germacrenes (Fig. 9b). When FDP was omitted from the reaction or when crude *E. coli* extracts of cells not expressing this gene were used, no sesquiterpene products were detected (data not shown).

Discussion

The epidermal glandular trichomes are considered the primary site of resin production in *C. creticus*. Many plants producing terpenes bear on their aerial organs a great population of glandular trichomes. Young leaves have both developing and mature glandular trichomes (Bosabalidis and Skoula 1998), whereas fully expanded leaves only mature glandular trichomes (Werker et al. 1993). The density of glandular trichomes decreases with leaf growth (young leaves have a denser pubescence). This might be an adaptive mechanism, wherein the young leaves, most tender and appetizing to herbivores, are given the highest protection (many secretions of glandular trichomes are deterrent or toxic to insects). Density of glandular trichomes is further considered to be associated with transpiration, leaf overheating, UV-B radiation, etc. (Wagner et al. 2004). The number of glandular trichomes

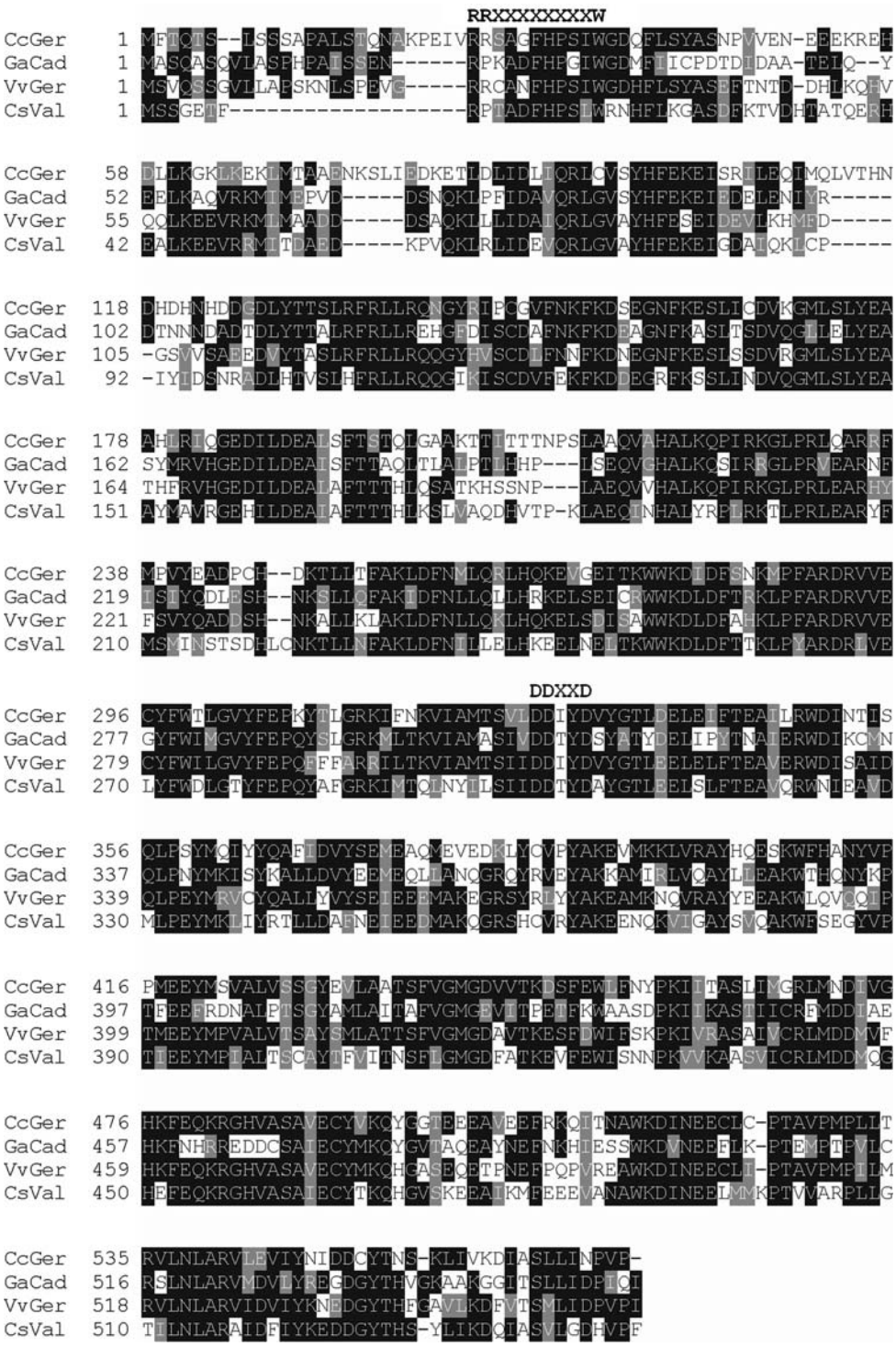

Fig. 8 Sequence comparison of the CcGer with other plant known sesquiterpene synthases. Alignment of deduced amino acid sequences of *Cistus creticus* sesquiterpene synthase (CcGer) with *Vitis vinifera* germacrene D synthase (VvGer, Genbank accession number: AAS66357), *Citrus sinensis* valencene synthase (GaCad, Genbank accession number: AAQ04608) and *Gossypium arboreum* (+)-δ-cadinene synthase (CsVal, Genbank accession number: X96429). The alignment was created using ClustalW2 (www.ebi.ac.uk/cgi-bin/clustalw2/) software and shading with BoxShade 3.21 software (www.ch.embnet.org/)

on the leaves is linearly associated with the yield in terpenes. Thus, the greater the number of glandular trichomes on the leaves, the higher the amount of terpene substances derived from them by distillation (Bosabalidis and Kokkini 1997). This is due to the fact that the glandular trichomes are the main leaf sites of terpene biosynthesis and possess the complete enzymatic equipment (Mc Caskill and Croteau 1995).

Chemical analysis of *C. creticus* subsp. *creticus* trichomes revealed that sesquiterpenes and labdane-type diterpenes followed a different profile of accumulation: young leaves were richer in labdane-type diterpenes than

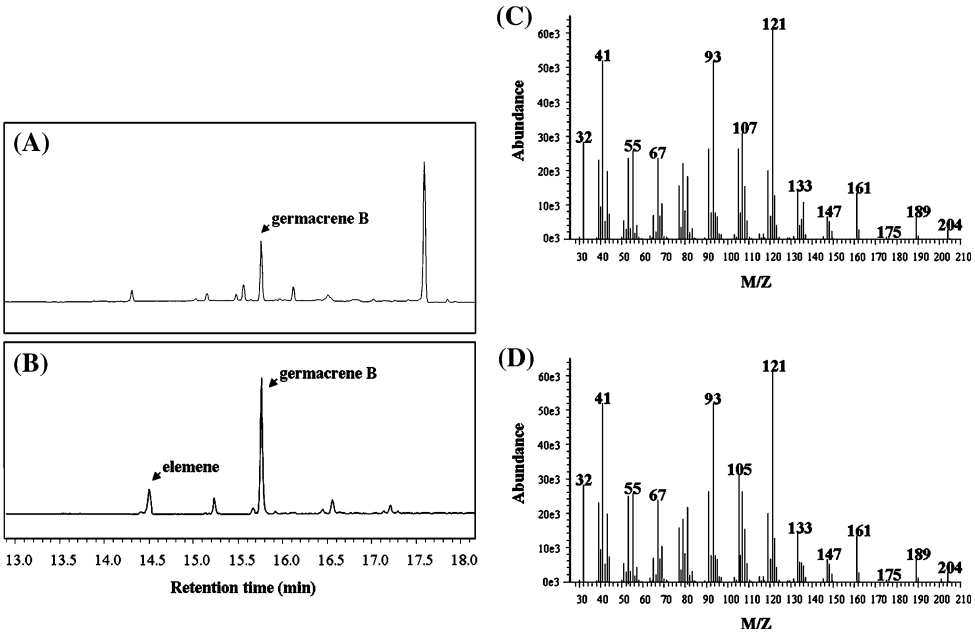

Fig. 9 Functional characterization of the CcGer encoding cDNA in *E. coli*. (**a**) Chromatograph of compounds extracted from leaves of wild tomato (*Solanum hirsutum*). (**b**) Chromatograph of reaction products of crude protein extracted from *E. coli* overexpressing clone incubated in assay buffer supplemented with farnesyl diphosphate. (**c**) Mass spectrum of enzymatic reaction product with RT = 15.78 min. (**d**) Mass spectrum of germacrene B

older ones, whereas older leaves contained higher amounts of sesquiterpenes compared to younger leaves, implicating a different control in accumulation of these compounds. This labdane-type diterpene accumulation during *C. creticus* leaf development resembled the expression pattern of geranylgeranyl diphosphate synthase genes, *CcGGDPS 1* and *2* at the mRNA and protein levels (Pateraki and Kanellis 2008). The observed decline in labdane-type diterpenes and the paralleled decrease in *CcGGDPS1* and *2* transcript levels (Pateraki and Kanellis 2008) in older leaves might be explained by the development of thick wax cuticle, which possibly makes the leaves less sensitive to biotic or abiotic stresses (Viougeas et al. 1995). On the other hand, the ample secretion of diterpenes in young leaves suggests a protective role in young tissues.

Transcriptome analysis of *Cistus creticus* subsp. *creticus* trichomes

A trichome-enriched cDNA library was constructed and an EST database of 2,022 clones (Accession numbers FF403916-FF405771 and FG347095-FG347260) was further analyzed. Assembly of the ESTs revealed relatively low redundancy, with more than 50% of the total number of ESTs found as singletons. This indicates similar expression levels for a large number of genes. The putative functions of the ESTs were further investigated by Blastx analysis, and putative functions were assigned according to MIPS FunCatDatabase. This analysis provided a rough global overview of expressed genes in *C. creticus* trichomes. A large proportion of the ESTs (38%) corresponded to proteins of unknown function and to proteins with no significant similarity to sequences deposited in nr protein database. These proteins are most likely involved in trichome-specialized functions. The percentage of non-annotated ESTs was equivalent to this of glandular trichome cDNA libraries from other species ranging between 22% and 35% (Aziz et al. 2005; Bertea et al. 2006; Gang et al. 2001; Lange et al. 2000).

The annotated ESTs (62%) were classified into 11 categories, according to their putative function. A significant proportion of the ESTs (13%) was assigned to the 'photosynthetic processes' class and mainly corresponded to ribulose biphosphate carboxylase/oxygenase (Rubisco) small subunit, photosystem I and II related and chlorophyll a/b binding proteins. Trichomes from several plant species i.e. *Mentha piperita*, *Sigesbeckia jorullensis* and *Saintpaulia ionantha* are photosynthetically active and fully developed chloroplasts are often present in both head and stalk cells (Heinrich et al. 2002; Saltveit and Hepler 2004; Turner et al. 2000; Wagner 1991). Furthermore the possibility of some contamination of mesophyll cells in the trichome preparation cannot be excluded. cDNA clones encoding various ribosomal proteins were also abundant and contributed to the 'protein synthesis'-related EST category being the second most prevalent (13%).

For the remaining ESTs that could tentatively be assigned a function the largest group comprised of secondary metabolism-related genes represented by 5% of total transcripts. The proportion of secondary metabolism related ESTs in *C. creticus* trichome cDNA library was lower than those observed in EST databases of mint and sweet basil glandular trichomes (Gang et al. 2001; Lange et al. 2000). The construction of the cDNA library from

glandular and non glandular trichomes could explain in part this phenomenon. Nevertheless, the relatively high abundance of genes encoding enzymes of secondary metabolism in this mixed trichome preparation (ranked third highest in terms of classes of genes) underlines the biosynthetic role of glandular trichomes (the 'basic metabolism' EST category was represented by an even slighter number of ESTs, 3%, in the EST collection). Among the secondary metabolism-related genes were terpenoid-, flavonoid-, other phenylpropanoid- and alkaloid-biosynthesis-related genes. Also, several cDNAs corresponded to acetyltransferases, methyltransferases, acyltransferases and hydroxylases; which are enzymes with high or low substrate specificity that catalyze the addition of various functional groups to basic skeletons of secondary metabolites. Three different cytochrome P450 genes related to secondary metabolism were found to be expressed in *C. creticus* trichomes accompanied by abundant cytochrome b5 transcripts coding for this indirect electron donor that is essential for the catalytic activity of cytochrome P450s (de Vetten et al. 1999).

The resin produced by *C. creticus* trichomes consists mainly of terpenoids and flavonoids (Chinou et al. 1994; Demetzos et al. 1994a, b, 1989) therefore, the presence of ESTs involved in their biosynthesis was as expected. However, the EST analysis revealed the presence of cDNAs in the library encoding enzymes participating in phenylpropanoid biosynthesis, an indication that low molecular weight phenylpropanoids may also contribute to the characteristic odor of *C. creticus* subsp. *creticus*. An interesting finding is the occurrence of alkaloid metabolism-related transcripts in *C. creticus* trichomes. Alkaloid compounds have not yet been characterized in *C. creticus* resin since chemical analysis, so far, has primarily focused on the identification of lipophilic terpenoid compounds.

Lipid metabolism-related genes are highly represented in the cDNA library (3%). Lipid transfer protein-related cDNAs have often been found to be highly abundant in glandular trichome libraries (Aziz et al. 2005; Gang et al. 2001; Lange et al. 2000; Teoh et al. 2006). In the case of alfalfa, one of these cDNAs was the most abundant transcript in the library represented by 8% of the clones (Aziz et al. 2005). Lipid transfer proteins are considered to participate in intracellular transport of lipophilic compounds through cellular membranes (Kader 1997). The intracellular trafficking can be proposed as their potential role in *C. creticus* trichomes where different cellular compartments (plastids, cytoplasm and endoplasmatic reticulum) are involved in the production of the lipophilic terpenoids.

Wax is part of the cuticle, the out-covering complex of the leaf epidermis that functions as a mechanical barrier against pathogen attack (Jarrold et al. 2007; Lequeu et al. 2003) but also serves to limit water losses (Riederer and Schreiber 2001). It is noteworthy that there is a greater surface to cell mass proportion for trichomes in comparison to other epidermal cells. Consequently, many of the genes in 'lipid metabolism' category were found to be related to wax biosynthetic pathways. Noticeably, a cDNA coding for a putative *CER1* (*ECERIFERUM1*) sterol desaturase involved in epicuticular wax biosynthesis was found to be among the ten most abundant transcripts in *C. creticus* trichomes. The observed severe trichome structural disorders in *Arabidopsis thaliana cer1* and *yre* double mutant—*YRE* is a gene homolog of *CER1* (Kurata et al. 2003)—underlines the important role of epicuticular waxes in normal trichome development and explains the high proportion of these transcripts in the trichome EST collection.

In *C. creticus* trichomes two different metallothionein genes were highly expressed and comprised 0.4% of total transcripts. Metallothionein genes have also been found to be predominantly expressed in the trichomes of other plant species and are believed to be involved in heavy metal homeostasis by sequestration of excess metal ions in the trichome's body (Foley and Singh 1994; Garcia-Hernandez et al. 1998). Glandular trichomes are known to accumulate and secrete various elements to the leaf surface (Broadhurst et al. 2004; Choi et al. 2001; De Silva et al. 2001; Kamiya et al. 2006; Kupper et al. 2000, 2001; Lavid et al. 2001; Salt et al. 1995). These observations are consistent with a putative detoxification role of trichomes and this could be the case for *C. creticus* trichomes as well.

Trichomes are also thought to regulate calcium apoplastic concentrations by secretion of calcium ions on their surface. Calcium is often used as a secondary messenger molecule in response to various environmental conditions (Plieth 2005). More precisely, unbound cytoplasmic calcium levels are of great importance for signal transduction pathways related to photosynthesis, stomatal opening and closure and plant responses to water deficit (De Silva et al. 2001). Trichomes as epidermal appendages are hypothesized to have a putative role in stomatal homeostasis (Martin and Glover 2007). Calmodulins are proteins that serve as calcium receptors and are implicated in plant growth and response to environmental stresses (Bouche et al. 2005). Plants usually have more than one gene coding for calmodulins (Boonburapong and Buaboocha 2007; McCormack et al. 2005). In *C. creticus* trichomes four genes coding for calmodulins were found to be transcribed together with two genes coding for Ca^{2+} pumps, thought to be involved in cytoplasmic calcium homeostasis (Kamiya et al. 2006). *C. creticus* is a drought-resistant Mediterranean shrub, thus reducing and controlling water losses represents one of its main functions.

Trichomes are extra-epidermal organs suited to overcome several biotic and abiotic stresses such as herbivore attack, pathogen infection, intense light radiation and high

temperatures. High concentrations of glutathione found in the trichomes are believed to play an essential physiological role in the protection of plants against oxidative stress caused in the above conditions as well as by heavy metal accumulation (Gutierrez-Alcala et al. 2000; Wienkoop et al. 2004). Glutathione serves as a cofactor for several enzymes such as glutathione peroxidases and lactoylglutathione lyases that are implicated in the detoxification processes in response to oxidative stress (Noctor et al. 2002). Detoxification also involves the conjunction of glutathione with cytotoxins by glutathione *S*-transferases that are eventually targeted into the vacuole (Dixon et al. 2002). Transcripts coding for all the above mentioned enzymes were found in great abundance in the *C. creticus* EST collection.

Trichome-specific genes

Subtractive microarray analysis was performed to determine the relative transcript levels of 1,248 trichome expressed genes between trichomes and a pool of tissues without trichomes: leaves, stems and roots. Fifteen putative trichome-specific genes were identified most of which are related to secondary metabolism, defence and detoxification. For six of these genes, RT-PCR experiments were performed to investigate their expression in various tissues of the plant. In the case of the putative sesquiterpene synthase, with an observed 12.5 fold change between trichome and trichome-free tissues, its transcripts were detected only in the trichomes and flower reproductive organs. In the other cases, four out of five genes corresponding to thaumatin-like protein, putative short-chain alcohol dehydrogenase, putative cytochrome P450 and protein of unknown function, revealed trichome preferential expression. Transcripts of the above genes in trichome-free tissues used for the subtraction were either absent or extremely low in tight correlation to microarray data. However, the observed expression of these genes in the flower tissues as evidenced by RT-PCR analysis suggests a trichome-favoured rather than trichome-specific expression.

Functional characterization of germacrene B synthase

Germacrene B is among the major volatile components in several essential oils from plants of pharmaceutical and/or ecological interest (Araujo et al. 2003; Eom et al. 2006; Salido et al. 2002; Singh et al. 2006). To date heterologously expressed *Solanum hirsutum* (former *Lycopersicon hirsutum*) SSTLE1 was the only sesquiterpene synthase found to convert farnesyl diphosphate to germacrene B as the major product (Van der Hoeven et al. 2000). In wild tomato germacrene B is constitutively produced and is thought to be part of the plant defence mechanisms (Carter et al. 1989a, b; Eigenbrode et al. 1994; Snyder et al. 1993). In *Cistus creticus* subsp. *creticus* germacrene B has been identified as the major product of the trichome-specific CcGer as revealed by the microarray and RT-PCR analyses. Trichomes function as a physical boundary between the plant and its environment and the localized production of germacrene B in these structures is hypothesized to be part of the primary line of chemical defence.

Acknowledgments We thank G. Papagiannakis for participating in the construction of *Cistus* microarrays; we are grateful to Dr. C. Sallaud (Librophyt, France) for providing the hexane extract of *Solanum hirsutum* and to Dr. M. Lazari for her advice during the *Cistus* hexane extractions. We acknowledge the help of E. Argyropoulou in isolating trichomes shown in Fig. 1b–e. This work was funded by GR-USA bilateral grant and the 99EΔ637 and 01EΔ416 research projects, implemented within the framework of the "Reinforcement Programme of Human Research Manpower" (PENED) and co-financed by National and Community Funds (25% from the Greek Ministry of Development-General Secretariat of Research and Technology and 75% from E.U.-European Social Fund). This work was also partially supported by grants to AKMON (AP.6260 EFA1250/17-5-2004) co-funded by 70% from the European Union Regional Development Fund and by 30% from the Operational Programme Competitiveness of the Greek Ministry of Development.

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