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Isolation and functional analysis of two *Cistus creticus* cDNAs encoding geranylgeranyl diphosphate synthase

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Abstract

Cistus creticus ssp. *creticus* is an indigenous shrub of the Mediterranean area. The glandular trichomes covering its leaf surfaces secrete a resin called "ladanum", which among others contains a number of specific labdane-type diterpenes that exhibit antibacterial and antifungal action as well as *in vitro* and *in vivo* cytotoxic and cytostatic activity against human cancer cell lines. In view of the properties and possible future exploitation of these metabolites, it was deemed necessary to study the geranylgeranyl diphosphate synthase enzyme (GGDPS, EC 2.5.1.30), a short chain prenyltransferase responsible for the synthesis of the precursor molecule of all diterpenes. In this work, we present the cloning, functional characterisation and expression profile at the gene and protein levels of two differentially expressed *C. creticus* full-length cDNAs, *CcGGDPS1* and *CcGGDPS2*. Heterologous yeast cell expression system showed that these cDNAs exhibited GGDPS enzyme activity. Gene and protein expression analyses suggest that this enzyme is developmentally and tissue-regulated showing maximum expression in trichomes and smallest leaves (0.5–1.0 cm). This work is the first attempt to study the terpenoid biosynthesis at the molecular level in *C. creticus* ssp. *creticus*.

Keywords: *Cistus creticus* ssp. *creticus*; Secondary metabolism; Terpene biosynthesis; Labdane diterpenes; Geranylgeranyl diphosphate synthase (GGDPS)

1. Introduction

Cistus creticus ssp. *creticus* is a perennial, Mediterranean shrub, found mainly in degraded areas like maquis and garigues ecosystems. Its leaf surface is covered with non glandular (stellate) and glandular, long, ball-headed, multi-cellular trichomes (Gulz et al., 1996). The resin or "ladanum" excreted from these glands was known and used since ancient times for its officinal and aromatic properties (Demetzos et al., 1990; Gulz et al., 1996). These traditional applications were corroborated by recent findings showing that resin's labdane-type components such as *ent*-3β-acetoxy-13-epi-manoyl oxide, *ent*-13-epi-manoyl oxide, (13*E*)-labd-13-ene-8a,15-diol, (13*E*)-labd-7,13-diene-15-ol, (13*E*)-labd-7,13-diene-15-yl acetate, (13*E*)-labd-7,13-dienol, sclareol [(13*R*)-labd-14-ene-8,13-diol] or manoyl oxide derivatives, in free form or incorporated in liposomes, exhibited strong *in vitro* cytotoxic and cytostatic activity against human cancer lines (Demetzos et al., 1994; Dimas et al., 1998, 1999, 2001, 2006; Matsingou et al., 2005, 2006). Further, sclareol in liposomal formulation reduced the growth rate of human colon cancer tumors (HCT116) developed in SCID mice (xenografts) via apoptosis and cell cycle arrest without any significant side effects (Dimas et al., 2007; Hatziantoniou et al., 2006). The above findings propose that sclareol or other labdane-type diterpenes incorporated into liposomes may possibly evolve into novel chemotherapeutic agents for the treatment of several types of human cancer. Within this context, an insight into the molecular mechanism governing the labdane diterpenes

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biosynthesis in *C. creticus* would be of great interest, in order to make possible future applications and increased production of these metabolites.

Diterpenes are produced from the condensation of dimethylallyl diphosphate (DMADP) with three units of isopentenyl diphosphate (IPP) (Koepp et al., 1995; Liang et al., 2002; Ogura and Koyama, 1998; Trapp and Croteau, 2001). The acyclic molecule (C20) produced, GGDP (geranylgeranyl diphosphate), undergoes a range of cyclisations to produce the parent skeletons of all diterpenoids. These reactions are catalyzed by diterpene synthases (or cyclases) and are followed by a variety of modifications of the parent skeleton to produce the many thousands of different plant-origin diterpenes (Bohlmann et al., 1998; Pichersky and Gang, 2000; Trapp and Croteau, 2001). Many metabolites, playing essential roles in plant physiology such as carotenoids, chlorophyll and tocopherol side chains, phytol, the plant hormones gibberellin and abscisic acid are synthesized through the diterpenoid biosynthetic pathway.

GGDPS (geranylgeranyl diphosphate synthase), is an homodimeric, short chain *trans*-prenyltransferase responsible for the synthesis of GGDP, and occurs nearly ubiquitously in plants, animals and bacteria (Ogura and Koyama, 1998). It is a branch point enzyme, which regulates in coordination with the other prenyltransferases (GDP and FDP synthase respectively) the precursor flux towards mono-, sesqui-, and diterpenoids production (Hefner et al., 1998; Laskaris et al., 2000). Consequently, this may affect the ratio of the produced mono-/sesqui-/di-terpenes. Thus, in view of the key role of this enzyme in diterpenes biosynthesis, the isolation and characterization of its cDNAs deserves further attention.

GGDPS cDNAs have been cloned and characterized from several plant species [e.g. *Arabidopsis thaliana* (Okada et al., 2000), *Taxus canadensis* (Hefner et al., 1998), *Scoparia dulcis, Croton sublyratus* (Kojima et al., 2000), *Hevea brasiliensis* (Takaya et al., 2003), *Helianthus annuus* (Oh et al., 2000) or *Solanum lycopersicon* (Ament et al., 2006)]. GGDPS seems to be encoded by a multigene family in many plant species studied until today. This is obvious from the submissions in the nucleotide (GenBank, EMBL-Bank, RefSeq, PDB) and protein (SwissProt, PIR, PRF, PDB, UniProt, InterPro) sequence databases. In tomato, for example, three different cDNAs have been sequenced until today (accession numbers: DQ159949, Q1A7T0, Q1A7S9), in *Adonis palaestina* three (accession numbers: AY661706, AY661707 and AY661708), in *Oryza sativa* three more (accession numbers: AK120768, Q6ET88 and Q9XHX1), while in *Daucus carota* only two (accession numbers: AB027705 and DQ192185). In *Arabidopsis*, based on the genome sequence, there are 12 putative GGDPS genes (Lange and Ghassemian, 2003). *Arabidopsis* and tomato are the only plant species where this multigene family has been studied and the differential expression, as well as the subcellular localization of specific members has been reported (Ament et al., 2006; Okada et al., 2000).

To investigate the early steps in the biosynthesis of diterpenes and their regulation in *C. creticus* ssp. *creticus* plant, two cDNAs encoding GGDPS (*CcGGDPS1* and *CcGGDPS2*) were cloned and functionally characterized. Moreover, the levels of the transcripts and peptide accumulation were studied in several plant tissues. The results indicate that this enzyme is developmentally and tissue-regulated showing maximum expression in trichomes and in smallest leaves (0.5–1.0 cm).

2. Results and discussion

2.1. Cloning of two full-length CcGGDPS cDNAs

Plant GGDPSs are encoded by multigene families while the genes that comprise these families seem to be differentially regulated and localized in different cellular compartments, like endoplasmic reticulum, plastids or mitochondria (Ament et al., 2006; Okada et al., 2000). In spite of the extended studies of the GGDPS genes in many plant species, only few of the isolated cDNAs have been functionally characterized.

Two cDNAs corresponding to *C. creticus* GGDPS genes, according to sequence comparison, *CcGGDPS1* and *CcGGDPS2*, were isolated using 5′ and 3′ RACE techniques (accession numbers: AF492022 and AF492023, respectively). *CcGGDPS1* full-length cDNA has a size of 1456 bp and encodes a deduced protein of 363 amino acids. It contains a 5′ untranslated region (UTR) of 167 bp and a 3′ UTR of 195 bp. The *CcGGDPS2* full-length cDNA has a size of 1311 bp and encodes a deduced protein of 369 amino acids. The 5′ UTR is 115 bp while the 3′ UTR has a size of 86 bp. A putative polyadenylation signal was found only in the *CcGGDPS2* (AATGAA), 23 nucleotides upstream of the polyA, while no polyA signal was found in the *CcGGDPS1* cDNA, as is the case with many plant transcripts (Graber et al., 1999). Pairwise comparisons of the putative CcGGDPS peptides with other known GGDPS revealed that they are highly similar with other plant GGDPS in a range of 73–53%, while the similarity with other non-plant eukaryotic and prokaryotic homologues is considerably lower (Table 2). The similarity between the *C. creticus* GGDPSs is 74% at the amino acid level.

In silico analysis, using the ChloroP v1.1 (Emanuelsson et al., 1999), TargetP 1.1 (Emanuelsson et al., 2000) and the Predotar V1.03 (Prediction of organelle targeting sequences, http://genoplante-info.infobiogen.fr/predotar/) programs showed that both CcGGDPS proteins contain a putative transit peptide that directs the mature protein into the plastids, with high probability (in a range of 57–97%). For the prediction of the cleavage site, the above programs lack consistency. However, it has been proven experimentally for the *Taxus canadensis* GGDPS that the GGDPS transit peptide extends till the region where the high conservation begins (Hefner et al., 1998). Likewise, the transit peptides for CcGGDPS1 and CcGGDPS2 pro-

teins are presumed to extend from Met1 till Phe70 and Phe76 amino acids, respectively (Fig. 1). Consequently, the putative mature CcGGDPS1 and CcGGDPS2 proteins consist of 294 aa and have a size of 31.7 kDa. It is worthwhile to notice that the putative mature CcGGDPS1 and CcGGDPS2 proteins have 85% identity, in pair wise comparison using the "Blast 2 sequences" (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi) program.

Among the several conserved regions in all *trans*-prenyltransferases there are two aspartate rich regions [DDxx(xx)D], the FARM (First Aspartate Rich Motif) and the SARM (Second Aspartate Rich Motif) located in the enzymes' large, central, hydrophobic cavity responsible for substrate binding and catalysis (Liang et al., 2002; Wang and Ohnuma, 1999). It has been shown, for the FDP synthase, that the FARM is bound to the allylic substrate – GDP – while the SARM is the binding site of the IPP (Koyama et al., 1994, 1995; Tarshis et al., 1996). Substitution of any of the aspartate molecules in those motifs, using site-directed mutagenesis, with different amino acids resulted in reduced enzymatic activity (for review, see Liang et al., 2002). In addition to the above-mentioned motifs, there is another characteristic region in these enzymes, the Chain Length Determination region (CLD), which is responsible for the size of the resultant product. This region contains the FARM domain and five amino acids upstream of it. According to Wang and Ohnuma (1999), the length of the final product is determined from the size of the fourth and fifth amino acids upstream the FARM, as well as from the type of the FARM motif

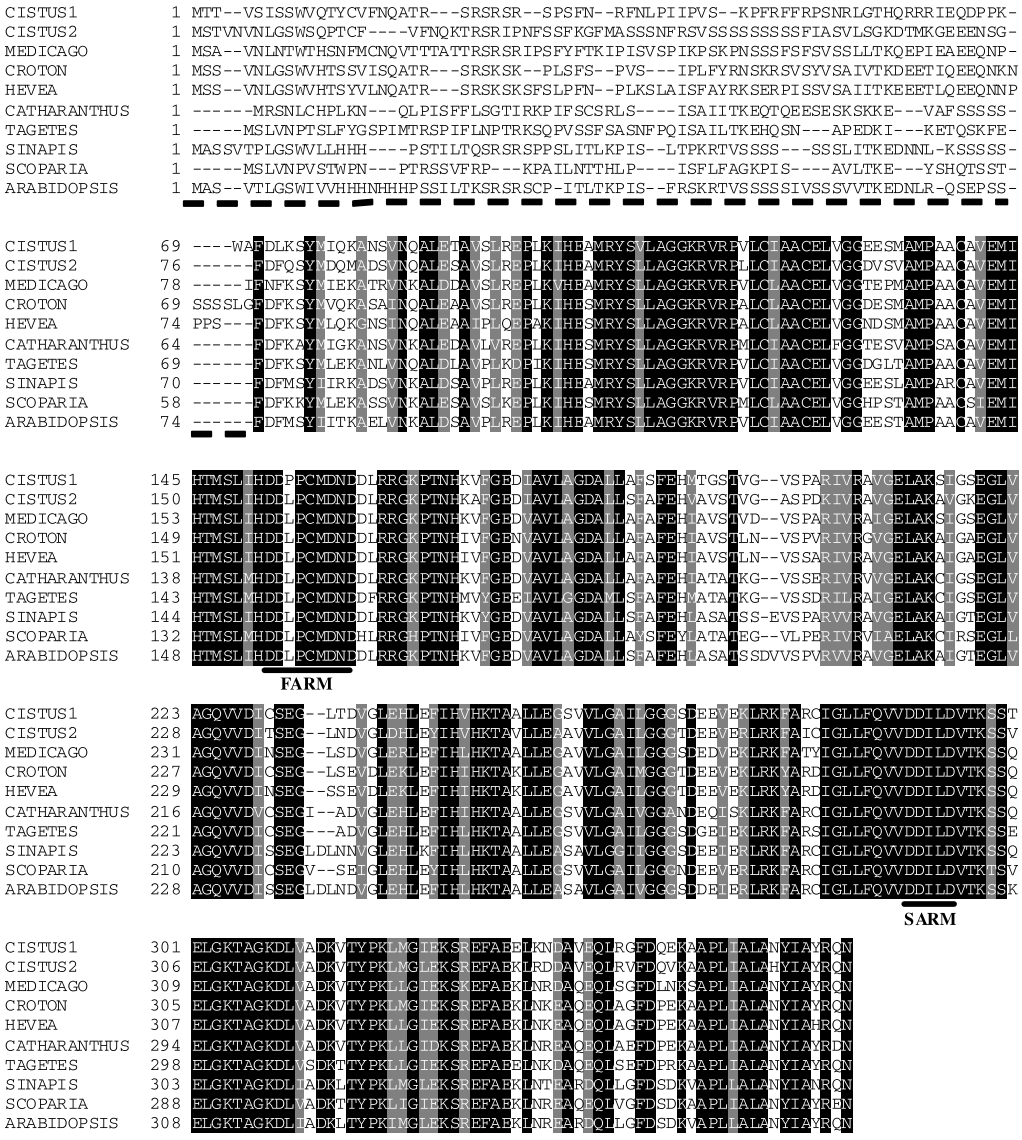

Fig. 1. Alignment of CcGGDPS with other plant proteins. Conserved regions like FARM and SARM motifs are underlined with a bold solid line, while the hypothetical transit peptide is underlined with a bold dashed line. Conserved amino acid residues in all the sequences used in this alignment are black boxed, while similar amino acids are in gray boxes. Alignments were performed with the ClustalW program (http://www.ebi.ac.uk/clustalw/) while the shading was done with the BoxShade 3.21 program (http://www.ch.embnet.org/software/BOX_form.html/).

(DDxxxxD or DDxxD). FDP and GGDP synthases are classified in several types according to this region (Wang and Ohnuma, 1999). In the case of plant and eubacterial GGDPS (type II GGDPS) the FARM has the DDxxxxxD motif (while the GGDPS from all other organisms do not have the two amino acid insertion). In these proteins the fourth and fifth position before the FARM are both devoid of aromatic amino acids (unlike the GDPS, the eukaryotic FDPS and the GGDPS from archaea) (Bouvier et al., 2005; Wang and Ohnuma, 1999). Both isolated cDNAs from the *C. creticus* contain the FARM (DDxxxxD) and SARM domains and seem to be classified within the type II of GGDPS, according to the CLD region sequence (Fig. 1). The fact that the plant and eubacterial GGDPS are classified in the same GGDPS type and that the DDxxxxD motif is found in plant and bacterial GDPS and GGDPS as well as in bacterial FDPS enzymes shows the common origin of these enzymes (Bouvier et al., 2005; Wang and Ohnuma, 1999). In the phylogenetic tree shown in Fig. 2 it is also evident that the deduced amino acid sequences of CcGGDPS1 and CcGGDPS2 are clustered together with the plant GGDPS.

Chen et al. (1994) proposed that all GDPS, FDPS and GGDPS originate from a common "ancient" prenyltransferase, which later evolved in all the short chain prenyltransferases. He proposed that this ancestral prenyltransferase segregated in two clusters, regardless of the chain length of their final product, the first for the eubacterial and the second for the eukaryotic enzymes, while the modifications responsible for the product length evolved independently, after the eukaryote and bacterial enzymes segregation (Chen et al., 1994). From the phylogenetic tree shown in Fig. 2, it is obvious that all eukaryotic (plants included) FDP synthases (type I) form a distinct cluster. The GGDPS from other eukaryotic organisms (animals and fungi) are segregated separately, while the plants' GDPS and GGDPS, which are plastidial enzymes, are grouped together with the bacterial prenyltransferases, supporting the notion of their common origin, through endosymbiotic phenomena. It is interesting to point out the fact that the plant FDPS, which is a cytoplasmic enzyme, segregates with the rest of the eukaryotic enzymes. Moreover, it is worthwhile to notice that several Arabidopsis GGDPS enzymes which have been reported as cytoplasmic enzymes (Okada et al., 2000) segregate along with the plastidial and bacterial GGDPS. Regarding the plant GGDPSs, it is apparent that peptides coming from the same species are not clustered necessarily together, unlike the CcGGDPSS1 and CcGGDPS2, but separately, probably according to their different role in the plant cell. However, as CcGGDPS1 and CcGGDPS2 are grouped together, this analysis does not provide any information regarding their functional role in the *C. creticus* cells or tissues.

As it was mentioned previously, plant GGDPSs are encoded by multigene families, comprising of more than two members in many plant species. In order to isolate other potential GGDPS cDNAs from *C. creticus*, 150 cDNA fragments, amplified by RT-PCR and highly degenerate primers using leaf trichome and leaf total RNA, were

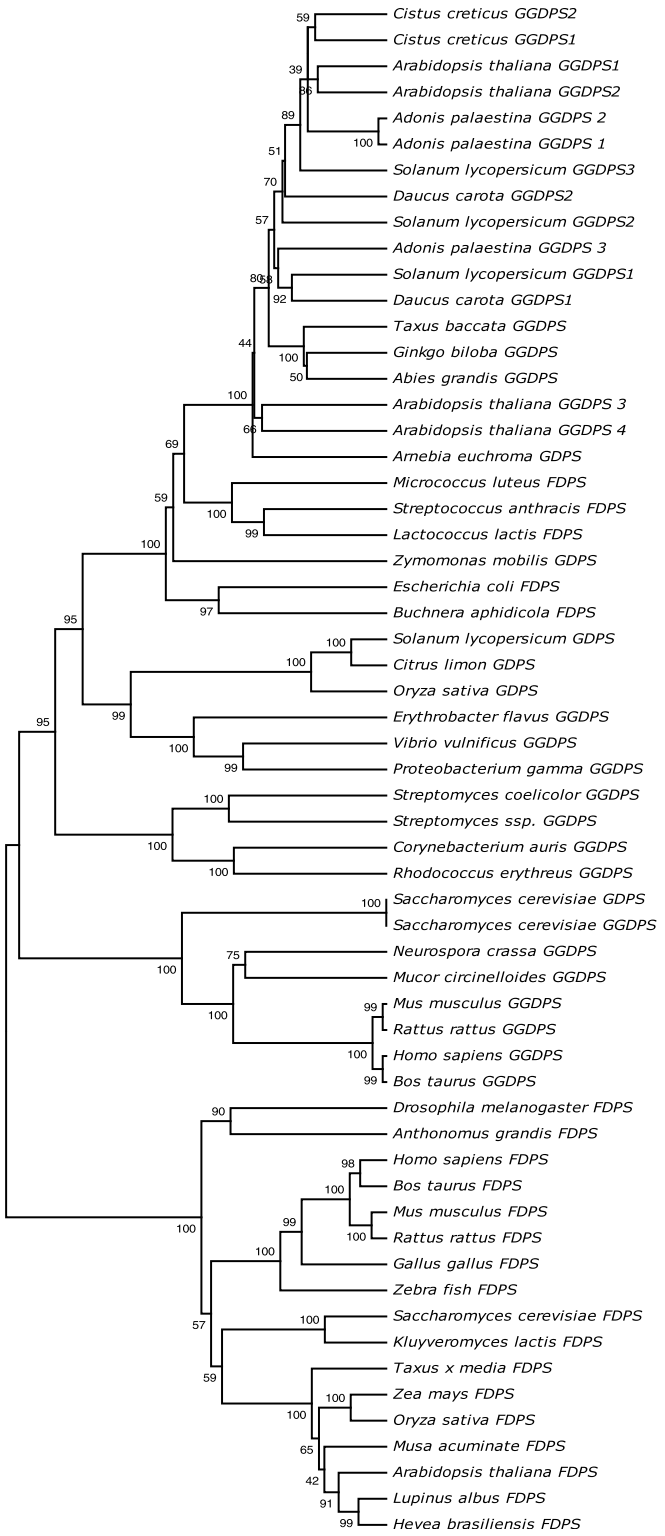

Fig. 2. Phylogenetic relationships of short chain prenyltransferases based on their amino acid sequences. The phylogenetic tree was constructed with the Neighbor-Joining method. The numbers indicated are the bootstrap values. The species from which the enzymes were obtained are indicated.

analyzed by sequencing and/or restriction patterns (data not shown). All the above fragments corresponded to the *CcGGDPS1* or *CcGGDPS2* genes and it was not possible to isolate a third GGDPS cDNA. From the Southern blot analysis (Fig. 3) it is obvious that there are more than two hybridization bands in the lanes RV and RI, while there are only two in the BG. The hybridization was performed with a radiolabeled conserved fragment of the CcGGDPS1 cDNA (from 618 nt till 1094 nt), which contains the second aspartate-rich motif. Therefore, it is possible that certain bands visualized in the blot (specially the more faint ones) correspond to genes encoding prenyltransferases other than GGDPS. Of course, one cannot exclude the possibility of the occurrence of some more GGDPS genes in the *Cistus* genome. From the results described above it can be assumed that *C. creticus* GGDPS is encoded by at least two genes.

2.2. Production of polyclonal antibodies against CcGGDPS protein

For analyses of the GGDPS enzyme at the protein level, a polyclonal antibody against the CcGGDPS was produced. For this purpose, a 192-amino acid peptide from the CcGGDPS1 (spanned from 171 aa till the end), 25 kDa in size, which had 84% identity with the CcGGDPS2 enzyme, was used as antigen for rabbit immunization. Western blot analyses on total *Escherichia coli* protein extracts from cells expressing the recombinant CcGGDPS1 protein and on total protein extracts from *C. creticus* leaves revealed that the resultant rabbit antiserum was able to recognize the recombinant, as well as the native CcGGDPS1 protein, while no cross-reaction with other *E. coli* or *C. creticus* proteins was observed (Fig. 4). In addition, the above antiserum was able to recognize the recombinant CcGGDPS2 protein expressed in the ANY119 yeast cells (Fig. 5).

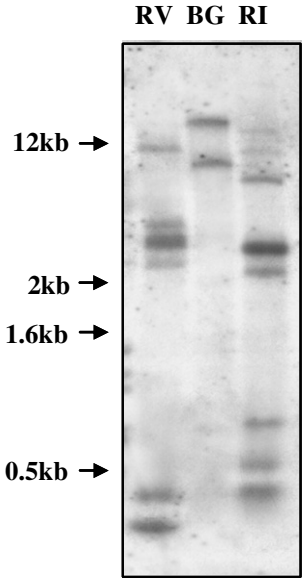

Fig. 3. Southern blot analysis. Genomic DNA (~30 μg) isolated from *Cistus creticus* leaves was digested with *Eco*RV (RV), *Bgl*II (BG) and *Eco*RI (RI), fractionated in 0.8% agarose-TAE gel, transferred to Hybond N-membrane and hybridised with a (α^{32}P)CTP-labeled fragment corresponding to a conserved region of the *CcGGDPS1* cDNA. Molecular weights are indicated at the left.

2.3. Functional characterization of CcGGDPS cDNAs

The ANY119 *Saccharomyces cerevisiae* mutant (*MATa*, *bet2-1*, *ura3-52*, *his4-619*), which is deficient in the β-subunit of type II geranylgeranyl transferase, *BET2* gene (Jiang et al., 1995), was used to functional characterize the *CcGGDPS* cDNAs. The *bet2-1* gene is a recessive temperature-sensitive mutant allele that inhibits yeast cell growth at 37 °C (Jiang et al., 1995). This system has been used before to demonstrate the function of putative GGDP synthases from *Taxus canadensis* (Hefner et al., 1998). Although yeast GGDP does not exhibit any GTTase activity, the increased GGDP cellular pool resulted from GGDPS gene overexpression, compensates for the low GGDP affinity of the mutated GTTase (Jiang et al., 1995). Based on these observations, several plasmid constructs, containing CcGGDPS1 and CcGGDPS2 cDNA fragments, were expressed in the ANY119 cells. Their growth was monitored under different temperatures (25 and 37 °C) and growth media (Fig. 6), in order to examine the *CcGGDPS1* and *CcGGDPS2* cDNA capability to complement the *bet2-1* mutation.

Three different fragments for each cDNA were used for plasmid construction, as described in Section 3: the pFL1 and pFL2 expressing the presumptive pre-mature or full-length proteins, the pM1 and pM2 coding for the hypothetical mature proteins and pT1 and pT2 expressing truncated version of the proteins. As mentioned above, both CcGGDPS1 and CcGGDPS2 contain a transit peptide

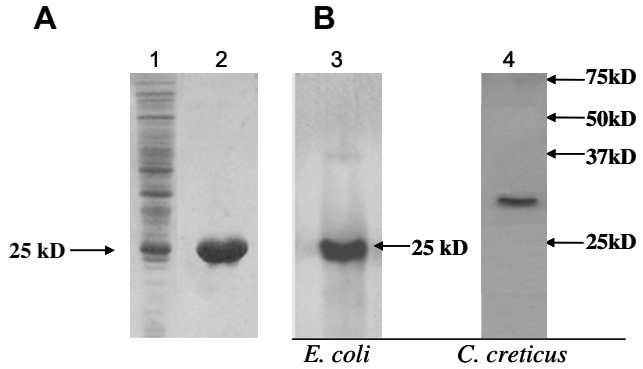

Fig. 4. Protein blot analysis of recombinant and native CcGGDPS proteins. (A) 12% SDS–PAGE stained with Coommassie Brilliant Blue R-250. Lane 1: total protein extract from *E. coli* cells expressing the recombinant CcGGDPS1 peptide; Lane 2: recombinant CcGGDPS1 protein purified from Ni-NTA-Agarose column, used as antigen. (B) Immunodetection of GGDPS protein. Lane 3: total protein extracts from *E. coli* cells expressing the recombinant CcGGDPS1 peptide; Lane 4: total protein extracts from *Cistus creticus* leaves (antiserum dilution 1:1000). Protein molecular weights are indicated in the right.

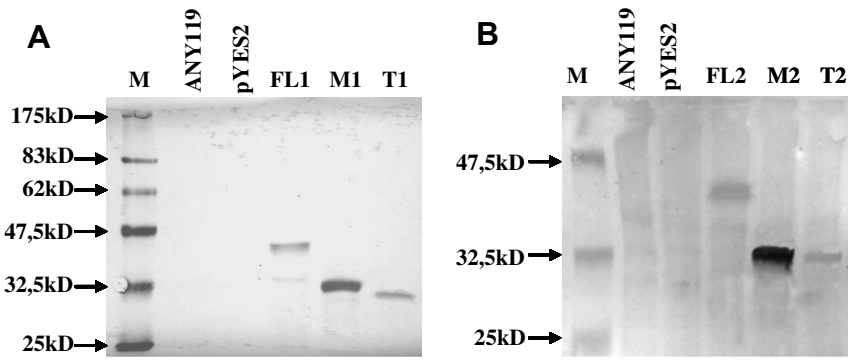

Fig. 5. Immunodetection of the recombinant CcGGDPS1 (panel A) and CcGGDPS2 (panel B) proteins expressed in the ANY119 cells. The constructs hosted in ANY119 cells are indicated in each lane. ANY119 cells without any construct (ANY119) or with the empty vector (pYES2) were used as controls (antiserum dilution 1:1000). The molecular weights of the protein bands are indicated on the left side. Equal amounts of total proteins were loaded in each lane (data not shown).

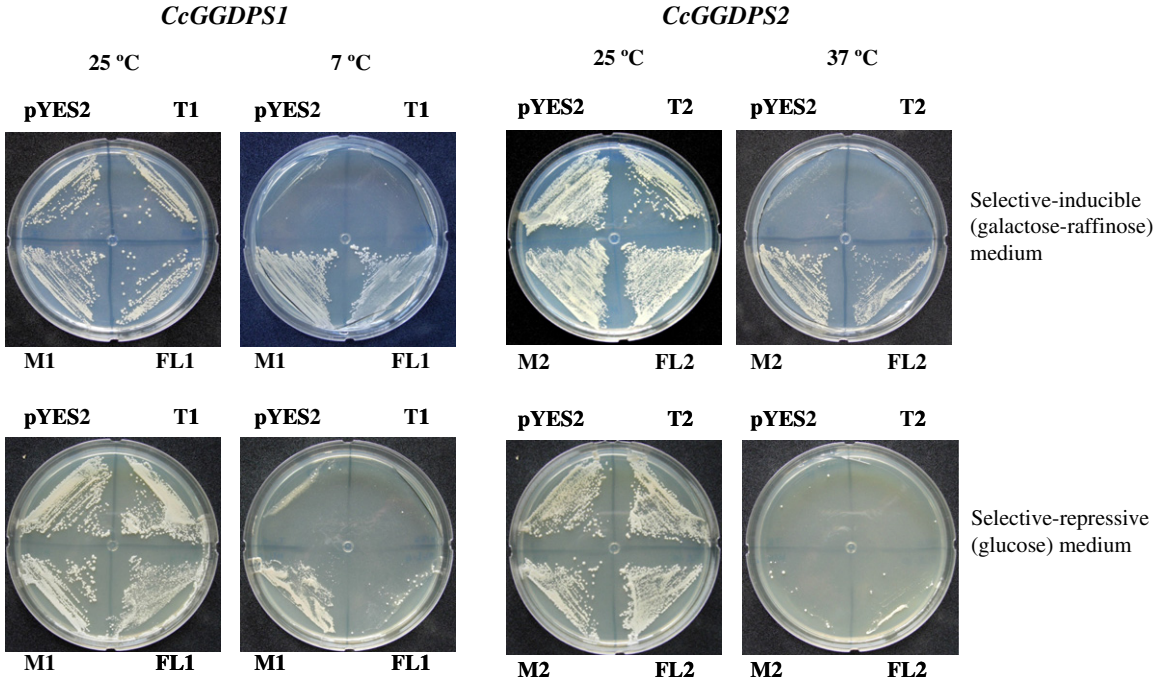

Fig. 6. Growth of ANY119 cells carrying the CcGGDPS1 and CcGGDPS2 constructs. The constructs hosted in each case are indicated above and below each Petri dish (pYES2, pFL1/2, pM1/2, pT1/2). The medium used is indicated on the right, while the incubation temperature is at the top.

presumably targeting them to the plastids. The length of this peptide was not possible to be accurately determined *in silico*. However, based on the notion suggested by Hefner et al. (1998) that a transit peptide can be considered the part of the protein expanded at the N-terminal non-conserved region, the transit peptides for CcGGDPS1 and CcGGDPS2 were regarded the regions of 69 and 75 aa in length, respectively, starting from the 5′ end (Fig. 1). The hypothetical transit peptides were removed from the inserts of the pM1/2 constructs to check their impact on the catalytic activity of the corresponding enzymes. The pT1/2 peptides expressed a truncated version of the CcGGDPS proteins, shorter than the hypothetical mature ones. It should be noted that the primers used for cloning the fragments of interest were designed in a way that a "Kozak motif" (ANN**ATG**G) was formed around the start-codon (ATG) of each recombinant transcript (Table 1), for effective translation (Kozak, 1987, 1991).

To ensure that the recombinant GGDPS proteins were efficiently expressed in the ANY119 cells hosting the above constructs, western blot analysis was performed in total yeast protein extracts. Fig. 5 demonstrates that the GGDPS antibody recognized all GGDPS proteins of the expected size (FL1: 39.2 kDa; M1: 31.7 kDa; T1: 30.9 kDa; FL2: 39.7 kDa; M2: 31.7 kDa; T2: 30.9 kDa). No bands were detected in lanes containing ANY119 cells with the empty vector, or ANY119 cells with no vector, suggesting that the CcGGDPS antibody recognized specifically the recombinant CcGGDPS proteins and not other yeast prenyltransferases. Moreover, all constructs were capable of

Table 1
Primers used for the amplification of the cDNA fragments used for the constructs inserted in ANY119 yeast cells

Primers used for CcGGDPS1 constructs	
GGDPS1FL.FOR1	5′ CC AAG CTT ATA **ATG** GGT ACT GTT AGC ATC AGT T CG 3′
GGDPS1TRM.FOR1	5′ CC AAG CTT ATA **ATG** GGT TTC GAT TTC AAG TCC TAC ATG 3′
GGDPS2TR.FOR1	5′ CC AAG CTT AAC **ATG** GTT CAG AAG GCG AAT TCC 3′
GGDPS1.REV1	5′ GC TCT AGA CTA ATT CTG CCT ATA AG C **AAT** G 3′
Primers used for CcGGDPS2 constructs	
GGDPS2FL.FOR1	5′ CC AAG CTT AAA **ATG** GGT ACT GTG AAT GTG AAT CTG G 3′
GGDPS2TRM.FOR1	5′ CC AAG CTT AAA **ATG** GGT TTT GAT TTC CAG TCT TAT ATG 3′
GGDPS2TR.FOR1	5′ CC AAG CTT AAG **ATG** GCG GAT TCC GTT AAT CAA GCG 3′
GGDPS2.REV1	5′ GC TCT AGA CTA GTT TTG CCT ATA AGC **AAT** G 3′

With bold are indicated the START (ATG) and the STOP codon, while the *Hin*dIII (AAGCTT) and *Xba*I (TCTAGA) restriction sites are underlined.

Table 2
Identity percentage of the *Cistus creticus* GGDP synthases with other known homologous enzymes

GGDP synthases (accession number)	Identity percentage (%) with the CcGGDPS1	Identity percentage (%) with the CcGGDPS2
Croton sublyratus (BAA86284)	73	71
Hevea brasiliensis (BAB60678)	73	70
Medicago truncatula (ABE80654)	72	70
Tagetes erecta (AAG10424)	71	67
Sinapis alba (CAA67330)	70	70
Scoparia dulcis (BAA86285)	70	66
Arabidopsis thaliana (Q0WUL9)	69	70
Catharanthus roseus (CAA63486)	67	67
Abies grandis (Q8W1R9)	57	59
Taxus × *media* (Q6Q291)	56	58
Oryza sativa (Q9XHX1)	53	54
Streptomyces coelicolor (NP_624521)	26	26
Mycobacterium tuberculosis (CAA17477)	24	24
Homo sapiens (NP_001032354)	22	21
Mus musculus (BAA76512)	21	20
Saccharomyces cerevisiae (AAB68296)	19	19

The peptide sequences were compared using the SMS2 program (Bioinformatics organisation, http://bioinformatics.org/sms2/ident_sim.html).

expressing all versions of the CcGGDPS proteins, but with different expression efficiencies. The pM1 and pM2 constructs resulted in higher protein expression levels compared with the rest of the constructs (Fig. 5A and B).

The growth of the ANY119 cells was monitored in two different synthetic culture media. The first one contained glucose which suppresses (minimize the activity) the GAL1 promoter that controls the expression of the recombinant GGDPS proteins, while the second one contained galactose and raffinose, which induce the GAL1. The growth of the ANY119 cells hosting the CcGGDPS constructs was tested at standard temperatures (25 °C), which permit normal growth of the *bet2-1* mutants and at high temperatures (37 °C), which restrict normal growth of the *bet2-1* mutants. ANY119 cells hosting the empty pYES2 vector were served as controls.

All ANY119 cells grew efficiently at 25 °C, irrespective of the construct or the medium used (Fig. 6). This was expected, since this temperature did not restrict the growth of these cells, even in the absence of BET2 protein. In contrast, only the cells expressing the FL1/2 and M1/2 peptides were able to grow at 37 °C in galactose-raffinose containing media, conditions that activated the GAL1 promoter and induced the recombinant CcGGDPS expression. No growth was observed in glucose, which suppressed the GAL1 activity. It is concluded, therefore, that only FL1/2 and M1/2 peptides coded for active GGDPS enzymes, since they were able to reverse the ANY119 high-temperature sensitivity phenotype. T1/2 truncated peptides did not complement GGDPS activity under these conditions. Cells expressing the mature GGDPS1 protein (M1) grew normally at 37 °C in media that suppressed the GAL1 promoter (Fig. 6). This was probably due to a minimum GAL1 activity under these conditions (and consequently a minimum M1 peptide synthesis), which apparently was sufficient for *bet2-1* complementation. This was consistent with the highest expression of M1/2 constructs as evidenced by the higher amounts of the corresponding polypeptides in western blots (Fig. 5). It seems that the first 69 and 75 aa of the CcGGDPS1 and 2 proteins, respectively, were not necessary for the enzymes' function, a fact that strengthens the hypothesis that these regions represented actual transit peptides. It is interesting to mention that the size of hypothetical CcGGDPS mature proteins (32 kDa) coincided with the size of the protein detected in the western blot of total leaf extracts from *C. creticus* leaves (Fig. 4B). The removal of 6 or 9 aa from the mature CcGGDPS1 and CcGGDPS2 peptides, respectively (constructs pT1 and pT2), was sufficient to abolish the GGDPS activity of the above proteins.

2.4. CcGGDPS gene transcripts and proteins tissue distribution

To study the regulation of CcGGDPS at the transcriptional and protein levels, RT-PCR was applied to detect specifically the *CcGGDPS1* and *CcGGDPS2* transcript levels and western blotting to analyse total CcGGDPS protein accumulation (Fig. 7). Roots and seeds exhibited very low expression of both genes. The mRNA steady state levels of *CcGGDPS1* and *2* genes were higher in leaf trichomes than in all other tissues studied (Fig. 7A). This corroborates

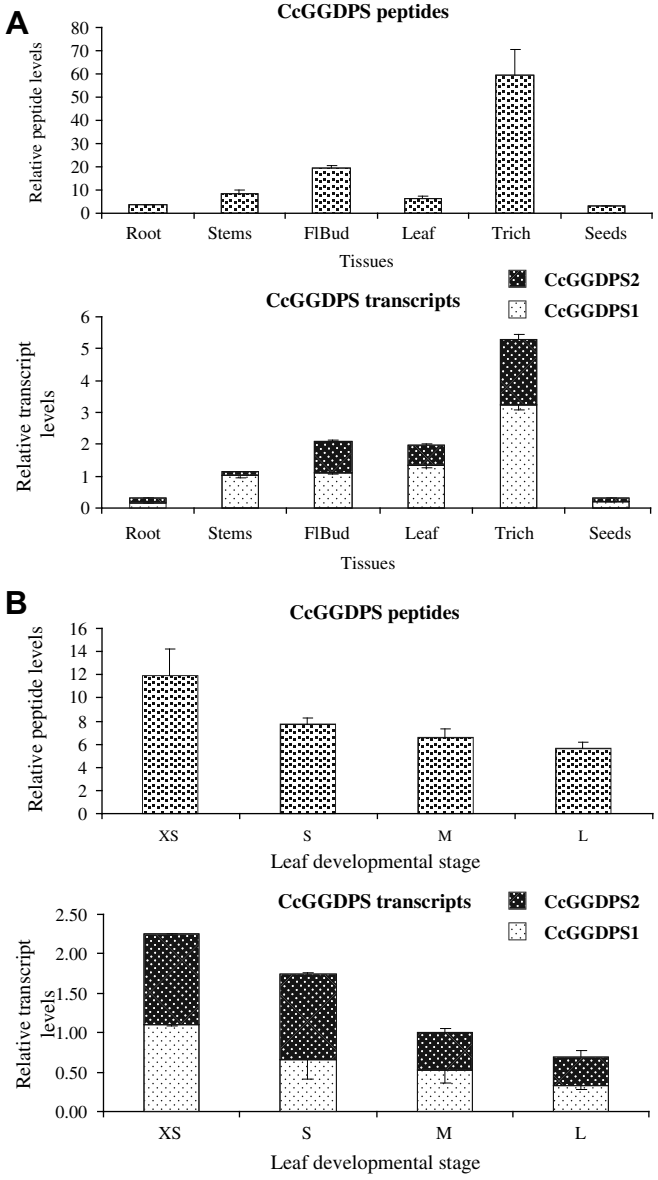

Fig. 7. Accumulation of GGDPS polypeptides and transcripts in several *Cistus* tissues (A) and in different leaf developmental stages (B) using protein blot and RT-PCR analyses, respectively. FlBuds: flower buds, Trich: trichomes. Leaf developmental stages: XS: leaves $\leqslant$1 cm; S: leaves 1–2 cm; M: leaves 2–3 cm; L: leaves $\geqslant$3 cm. Ten micrograms of total protein were loaded in each lane.

with findings showing that glandular trichomes is the site of terpenoid biosynthesis (Gang et al., 2001; Gershenzon et al., 1989, 1992). The results show also that the accumulation of the two *CcGGDPS* gene products followed different tissue pattern. This is an implication of the different roles of the two *GGDPS* genes could have in *Cistus* tissues. After trichomes, accumulation of *CcGGDPS1* was higher in leaves followed by the flower buds and stems, while *CcGGDPS2* was higher in flower buds and next in leaves. In terms of total *CcGGDPS* transcript steady state levels, leaves, flower buds and stems followed trichomes. The high levels of the *CcGGDPS* transcripts in the flower buds could be explained from the fact that increased needs for isoprenoids maybe needed for the protection of this sensitive tissue as well as the for the formation of the flower pigments and volatiles (Pichersky and Gang, 2000; Tholl et al., 2005). Differential expression of the GGDPS gene family members has also been reported for several genes in *Arabidopsis* (Okada et al., 2000) and for two in tomato (Ament et al., 2006).

In view of the evidence that GGDPS genes were differentially expressed in tissues studied, we next focused our study on the accumulation of CcGGDPS protein in the same tissues. Indeed, the levels of CcGGDPS immunoreactive polypeptides paralleled the expression of total GGDPS transcripts in the above tissues, with trichomes being the richest tissue in CcGGDPS protein. These findings suggest that the control of CcGGDPS synthesis is mainly at the transcription level.

2.5. Developmental regulation of CcGGDPS protein and transcripts

Initial studies indicated that *C. creticus* trichomes from very young (0.5–1.0 cm) and young leaves (1–2 cm) contained the highest amount of labdane-type diterpenes (Falara and Kanellis, unpublished results). Similarly, other investigators showed that the production of secondary metabolites in leaves and trichomes is under developmental control (Gershenzon et al., 2000; McConkey et al., 2000; Valkama et al., 2004). Therefore, it was deemed necessary to study the CcGGDPS protein and mRNA profile during *Cistus* leaf development. Leaves of different size ranging from extra small to large were harvested and total protein extracts analysed in terms of their immunoreactive CcGGDPS protein content (Fig. 7B). It is evident that CcGGDPS polypeptides' accumulation was inversely related to the size of the leaves, that is the smallest or youngest leaves expressed the highest amount of protein (Fig. 7B).

In order to examine if these differences were due to the variations in transcript levels and also to distinguish which member of the *CcGGDPS* gene family was responsible for this pattern, we monitored the level of the corresponding mRNAs in the same leaves by RT-PCR analysis (Fig. 7B). Total transcript accumulation of the two *CcGGDPS* genes paralleled the corresponding protein accumulation during leaf development, being the maximum expression of both genes in the extra small leaves. Thereafter, *CcGGDPS1* and *2* transcript abundance exhibited a progressive decline. However, it seems that both genes were expressed in relatively equal amounts during the whole period, suggesting a similar developmental and transcriptional control during leaf development. This *CcGGDPS1* and *2* expression pattern at the mRNA and protein levels resembled the labdane-type diterpenes' accumulation during *C. creticus* leaf development (Falara and Kanellis, unpublished data). Monoterpene accumulation and expression of their biosynthetic genes, for example in

peppermint (*Mentha* × *piperita* L.), although are under developmental control as in *C. creticus*, however, they follow a different pattern with maximum accumulation of compounds and the corresponding mRNA in medium size leaves aged close to 12-day of growth (McConkey et al., 2000). This disparity can be attributed either to different physiological functions of diterpenes and monoterpenes during leaf development or due to different type of trichome in these two species and the way that these compounds are stored and excreted. In peppermint, the peltate trichomes contain a storage cavity where these metabolites are stored (Turner et al., 2000), whereas the *C. creticus* trichomes do not store the produced metabolites, but they excrete them to the environment (Gulz et al., 1996). Similarly, Kuntz et al. (1992) showed that GGDPS gene is developmentally regulated in *Capsicum annum* leaves where the levels of the corresponding mRNAs are reduced with leaf age. Finally, the observed decline in *CcGGDPS1* and *2* transcript levels in older leaves (Fig. 7B) and the corresponding lower production of labdane-type diterpenes (Falara and Kanellis, unpublished data) might be explained by the substitution of terpenoids by a thicker wax cuticle which possibly makes the leaves less sensitive to pathogens, herbivores and other biotic or abiotic stresses (Viougeas et al., 1995).

In conclusion, it is shown that the functional characterised *CcGGDPS1* and *2* genes and the corresponding proteins are tissue and developmentally regulated and this control is exerted at the transcript level.

3. Experimental

3.1. Plant material

C. creticus ssp. *creticus* plants were grown outdoors, in the area of Thermi, Thessaloniki, Greece, at the premises of the National Agricultural Research Foundation. Plant tissue was collected, immediately frozen in liquid nitrogen, and stored at −80 °C.

3.2. Total RNA, genomic DNA purification and Southern blot analysis

Total RNA and genomic DNA from *C. creticus* tissues were extracted following the protocols described by Pateraki and Kanellis (2004). Genomic DNA (30 μg) was digested with *Eco*RV, *Bgl*II and *Eco*RI endonucleases, fractionated in 0.8% (w/v) agarose gel, transferred on to nylon membrane (Nytran® 0.45, Schleicher & Schuell) and hybridised with a radiolabeled *CcGGDPS1* fragment, corresponding to a highly conserved region of the gene. Probe labelling was carried out using the RadPrime DNA Labeling System (Invitrogen, Life Technologies, Madison, WI, USA). Blot hybridization, membrane washing and autoradiography were performed as described by Church and Gilbert (1984).

3.3. Cloning and sequence analysis of C. creticus GGDPS full-length cDNAs

An initial cDNA fragment, 470 bp, corresponding to a *C. creticus GGDPS* was amplified by PCR with degenerate primers [GGDP.SEN2: 5′ CA(CT) GA(CT) GA(CT) (CT)T(AGCT) CC(AGCT) TG(CT) ATGG 3′ and GGDP.AS1: 5′ (AG)TC (AGCT)TT (AGCT)CC(AGCT) GC (AGCT)GT (AGCT)TT (AGCT)CC 3′)] from *C. creticus* genomic DNA. For the cloning of the 5′ of the GGDPS cDNAs, 5′ RACE was performed using the "5′ RACE System for Rapid Amplification of cDNA Ends" (Invitrogen, Life Technologies, Carlsbad, CA) and the gene specific primer GGDP.AS3 (5′ GCC AAC TGT GGA CCC CGT CAT GTG CTC 3′). From this procedure two PCR fragments were produced, corresponding to two different *CcGGDPS* cDNAs. The 3′ of these cDNAs were obtained using 3′ RACE techniques with the primers: GGDP.SEN2, specGGDPS2.FOR (5′ ATT GCC GCG TGT GAG CTC GTC GGC GG 3′), and oligodT (5′ ACT AGT CTC GAG TTT TTT TTT TTT TT TTT TT 3′). As template for the 5′ and 3′ RACE reactions was used single strand cDNA synthesized using the oligodT primer and reverse transcriptase SuperScript II (Invitrogen, Life Technologies, Carlsbad, CA) from DNaseI-treated (Promega, GmbH, Mannheim, Germany) total RNA isolated from young leaves. PCR products were cloned into pGEM-T Easy (Promega GmbH, Mannheim, Germany) vector and sequenced using the LI-COR Long Read 4200 automated sequencer and the "Sequitherm EXCELII" kit (Epicentre, Madison, WI, USA). Phylogenetic analyses were performed using neighbor joining analysis by MEGA 3.1 program. Bootstrap values were calculated by distance analysis for 1000 replicates.

3.4. Antibody production against CcGGDPS1

For the antibody production, the CcGGDPS1 fragment from the Phe172 until the Asn363 amino acids was used as corresponded to a highly conserved and hydrophilic region. The GGDPa/b.FOR (5′ ATA CTC GAG TTC GGC GAG GAT ATC GCG GTG 3′) and GGDPa/b.REV (5′ ATC GGA TCC TCA TTC TGC CTA TAA GCA ATG 3′) primers, containing the *Xho*I and *Bam*HI restriction sites, respectively, were used for the amplification of the corresponding cDNA fragment. The fragment was cloned in the *E. coli* expression vector pET-16b (Novagen, Madison, WI, USA) and expressed in *E .coli* BL21(DE3) cells. The recombinant protein was 6XHIS-tagged and was purified from BL21(DE3) cells' protein extract, under denaturated conditions with 8 M Urea, using a Ni-NTA-Agarose (Novagen, Madison, WI, USA) column, according to the manufacturer's instructions. The purified recombinant protein (serving as antigen) was injected in rabbits for antibody production, in the facilities of the Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology, Heraklion, Greece. Antiserum of the rabbit was collected and stored at −80 °C.

3.5. Generation of GGDP synthase yeast expression constructs and complementation assays

PCR primers containing a *Hin*dIII (AAGCTT) or a *Xba*I (TCTAGA) site were designed based on the 5′ and 3′ termini of the *CcGGDPS1* and *CcGGDPS2* cDNAs (Table 1), and used for the amplification and cloning of the cDNA fragments in the pYES2 yeast expression vector (Invitrogen, Life Technologies Carlsbad, CA). Three fragments were amplified for each cDNA. The first one was designed to express the hypothetical full-length CcGGDPS proteins (pFL1 and pFL2 constructs), the second to express the hypothetical mature proteins (pM1 – from Phe70 till STOP codon-, and pM2 – from Phe76 till STOP codon-constructs) while the third one to express a truncated version of each protein (pT1 – from Met76 till STOP codon-, and pT2 – from Met85 till STOP codon-constructs). The fragments amplification was performed using the *Platinum Pfx* DNA polymerase, an enzyme with proofreading activity, (Invitrogen, Life Technologies, Carlsbad, CA) in order to minimize the possibility of sequence discrepancies. The constructs were verified with sequencing and inserted in ANY119 yeast cells, with the LiAc method (Becker and Lundblad, 1998). The CcGGDPS recombinant proteins were expressed in yeast cells under the control of GAL1 promoter of the pYES2 vector. *S. cerevisiae* strain ANY119 was a gift from Dr. S. Ferro-Novick (Howard Hughes Medical Institute, Yale University) (Jiang et al., 1995). Isolated colonies, corresponding to each construct, were streaked on plates with synthetic media containing glucose (2%, v/v) (suppress the GAL1 promoter) or galactose (2%, v/v) together with raffinose (1%, v/v) (induce the GAL1 promoter), as carbon source (lacking uracil) and grown for 3 days at 25 or 37 °C. For the GGDPS protein expression in liquid media, isolated colonies were used to inoculate media containing glucose (2%, v/v), cells were grown for 16 h and then collected, rinsed with sterile water and grown for four more hours in media containing galactose/raffinose (2% and 1%, v/v, respectively).

3.6. Protein extraction and western blot analysis

Total protein extraction from *C. creticus*: 250 mg of several plant tissues collected from plants growing outdoors were grinded and homogenized in 0.5 ml of extraction buffer (2% SDS, 0.2 M Tris–HCl, pH 8.0, 5 mM EDTA, 5 mM $MgCl_2$, 10% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 2 mg/ml PMSF, 3% (w/v) PVPP). The homogenate was incubated for 10 min at 94 °C and centrifuged for 10 min at 15,000*g*. The supernatant was collected and stored at −20 °C.

Total protein extraction from yeast: cells collected from 5 ml liquid culture were resuspended in 0.5 ml of yeast protein extraction buffer (1% NP40, 120 mM NaCl, 1 ml PMSF). Equal volumes of glass beats were added and vortexed for 20 min at 4 °C. The mixture was centrifuged for 5 min at 13,000 rpm and the supernatant was collected and stored at −20 °C.

Total protein extraction from *E. coli* cells and western blot analyses were performed according to Sambrook et al. (1989). Ten micrograms of total crude protein extracts were loaded in each lane shown in Fig. 7. The proteins of interest were detected using as primary antibody the anti-GGDPS, in dilution 1:1000, and as secondary a horseradish peroxidise conjugated goat anti-rabbit antibody (dilution 1:2000) and 3,3′ diaminobenzidine (DAB)/hydrogen peroxide.

3.7. RT-PCR reactions

RT-PCR techniques were used to monitor the expression of the *CcGGDPS1* and *CcGGDPS2* genes in *C. creticus* tissues. First strand cDNA synthesis was performed using an oligodT primer and M-MLV reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA) from DNaseI-treated (Promega, GmbH, Mannheim, Germany) total RNAs isolated from *C. creticus* tissues. The gene specific primers used for the PCR reactions are shown in Table 3. The constitutively expressed *C. creticus* Eukaryotic Translation Elongation Factor-1α cDNA (*CcEF1a*, accession number EF062868) was used as internal control. The cycling conditions were optimized for each pair of primers in a way that sharp bands corresponding to the expected fragments were amplified with out any signs of non-specific products. The PCR amplified products for the CcGGDPS1 cDNA was 612 bp, for the CcGGDPS2 was 261 bp, while for the CcEF1a was 411 bp. The fragments were cloned into pGEM-T Easy (Promega GmbH, Mannheim, Germany) vector and sequenced using a LI-COR Long Read 4200 automated sequencer and "Sequitherm EXCELII" kit (Epicentre, Madison, WI, USA) to verify the amlicon identity. Relative transcript levels were visualized by setting the cycle numbers so that the rate of PCR product amplification was in the early exponential stage of the reaction. PCR products were resolved in agarose gel electrophoresis, photographed and quantified with ImageJ program at http://rsb.info.nih.gov/ij/ (Abramoff et al., 2004).

Table 3
Primers used for the CcGGDPS1 and CcGGDPS2 expression studies

Gene	Primers	Sequence
CcEF1a	CcEF1a.For1	5′ GGT CCT ACT GGT TTA ACC ACT G 3′
	CcEF1a.Rev1	5′ CTC GGA GAA GGT CTC CAC AAC C 3′
CcGGDPS1	CcGGDPS1.For1	5′ AGT TGG CGT ATC CCC CGC CCG 3′
	CcGGDPS1.Rev1	5′ TAC ATC TGT CTC TAA GCA GTC GC 3′
CcGGDPS2	CcGGDPS2.For1	5′ GAT GTG ACG AAA TCT TCC GTG 3′
	CcGGDPS2.Rev1	5′ CTT TTA TGC TTC TTT CAT TCA TAG 3′

The experiments were performed in duplicates regarding biological replications, where each individual sample was a pool of approximately 10 plants. Two technical replications were performed for each biological replication mentioned.

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