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Cloning of casbene synthase cDNA: Evidence for conserved structural features among terpenoid cyclases in plants

(phytoalexins/castor bean/*Ricinus communis*/defense response)

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ABSTRACT **A near-full-length casbene synthase cDNA clone, pCS7, was isolated by using a partial cDNA clone, pCS4, to probe a λgt10 library constructed from poly(A)$^+$ RNA from elicited castor bean seedlings. The cDNA insert had a length of 1983 bases with a polyadenylate tail of 19 bases. Translation of the cDNA sequence revealed an open reading frame encoding a 601-aa protein with a predicted M_r of 68,960. Search of the GenBank data base with the deduced translation product revealed 42% identity and 65% similarity with 5-*epi*-aristolochene synthase from tobacco and 31% identity and 53% similarity with limonene synthase from spearmint. Each of the three proteins catalyzes an intramolecular cyclization of a prenyl diphosphate substrate to a specific cyclic terpenoid hydrocarbon product. The proposed reaction mechanisms for the three catalytic processes share common chemical features, even though the products being formed are members of three different classes of terpenoid compounds. Analysis of the alignment of the three proteins suggests that both primary and secondary structural elements are conserved. These similarities suggest that the genes that encode terpenoid cyclization enzymes of this type in angiosperms have undergone divergent evolution from an ancestral progenitor gene. In support of this proposition, the locations of five of the six introns in the casbene synthase gene align very closely with those of the five introns in the 5-*epi*-aristolochene synthase gene.**

Casbene is a macrocyclic diterpene hydrocarbon that serves as a phytoalexin in castor bean (*Ricinus communis* L.) (1). Casbene synthase (CS; EC 4.6.1.7) (previously designated casbene synthetase) from castor bean seedlings catalyzes the cyclization of geranylgeranyl diphosphate (GG*PP*) to form the macrocyclic hydrocarbon in a single enzymatic step. The inducible enzyme activity is found in proplastids of germinating seedlings infected by the fungus *Rhizopus stolonifer* (2). The plant cells recognize oligogalacturonide fragments released from pectic components of the plant cell wall by a secreted fungal endopolygalacturonase as a signal to activate CS induction (3). CS activity reaches detectable levels after 5 hr of incubation with pectic fragments, with a peak occurring after 10–12 hr (4). The active enzyme consists of a single polypeptide and has an apparent M_r of 59,000 as determined by SDS/PAGE. Polyclonal antibodies to the purified enzyme have been used to isolate a partial cDNA clone from a λgt11 library (5). RNA gel blots probed with this clone show that casbene synthase mRNA is undetectable in unelicited seedlings but accumulates to a maximum at 6 hr following elicitation. Nuclear run-on experiments indicate that control is exerted at the level of transcription.

A full-length CS cDNA clone was isolated as part of an effort to characterize the gene structure in order to identify cis-acting elements involved in the induction of this gene by oligogalacturonide elicitors. This report describes the characterization of this clone[§] and the comparison of the primary structure of CS deduced from it with the deduced primary structures derived from two other nucleotide sequences reported for higher plant terpenoid cyclization enzymes: 5-*epi*-aristolochene synthase (EAS), which is involved in the initial cyclization step for farnesyl diphosphate (F*PP*) that leads to sesquiterpenoid phytoalexins in tobacco (6), and (−)-4*S*-limonene synthase (LS), which catalyzes the cyclization of geranyl diphosphate (G*PP*) to essential oil components in spearmint (7). Significant similarities exist among the deduced primary structures of the three enzymes. This result implies that a similar overall conformation, and possibly the relative positions of similar side chain functional groups, is necessary for catalysis by this group of enzymes in plants. An abstract referring to some of this work has been published (8).

MATERIALS AND METHODS

Isolation of Nucleic Acids. Castor bean seeds were germinated aseptically (9) and treated with elicitor (5). Total RNA was isolated (10), and contaminating polysaccharides were removed (11). Poly(A)$^+$ RNA was isolated from the total RNA by two cycles of oligo(dT)-cellulose chromatography (Collaborative Research). The purified sample was precipitated with ethanol, and the resulting precipitate was resuspended in RNase-free water for storage at −70°C.

Library Construction and Screening. Double-stranded cDNA was synthesized with the SuperScript system (Life Technologies, Gaithersburg, MD) from 4 μg of poly(A)$^+$ RNA obtained from elicitor-treated seedlings. The cDNA was then fractionated on Sephacryl S-500 HR columns (Pharmacia LKB) to enrich for larger cDNA inserts. Eighty-five nanograms of size-fractionated cDNA was ligated to *Eco*RI/*Not* I adapters and then ligated to *Eco*RI-digested λgt10 vector DNA and packaged *in vitro* with Packagene extracts (Promega). Phage virions were plated on *Escherichia coli* MB406 (Promega), and duplicate nitrocellulose filter replicas were screened with the pCS4 insert (5) and 5′ sequences from the CS gene (unpublished data) that were labeled by random priming (12). The probes were chosen to identify a clone that should be full-length. Hybridization was for 18 hr at 42°C in 50% formamide/5× standard saline citrate (SSC)/0.1% SDS/50 mM sodium phosphate, pH 7, containing (100 heparin μg/ml) and yeast tRNA (20 μg/ml). Filters were washed for 1 hr at 42°C with three changes of preequilibrated 0.1× SSC/0.1% SDS. Plaques hybridizing to both probes were

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Abbreviations: CS, casbene synthase; EAS, 5-*epi*-aristolochene synthase; F*PP*, farnesyl diphosphate; GG*PP*, geranylgeranyl diphosphate; G*PP*, geranyl diphosphate; LS, limonene synthase.

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§The nucleotide sequence reported in this paper has been submitted to the GenBank data library (accession no. L32134).

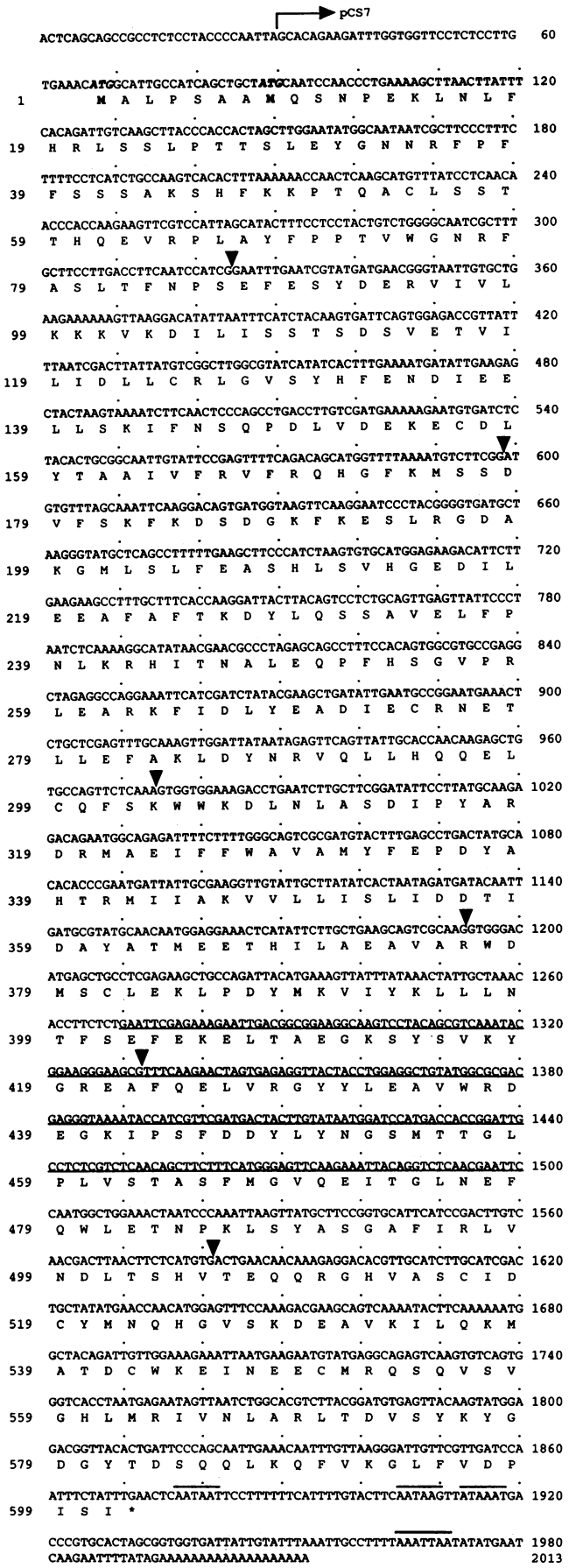

FIG. 1. The nucleotide sequence of pCS7 and the predicted sequence of the polypeptide it encodes. The nucleotide sequence is numbered on the right. The amino acid sequence is given in the single-letter code and is numbered on the left. Possible translation start sites are shown in boldface italics. pCS4 sequences are indicated by underlining. Putative polyadenylylation signal sequences are overlined. Bases 1–31 are derived from the genomic clone and results from primer extension experiments using poly(A)$^+$ RNA (unpublished observations). Intron locations are shown by an arrowhead (unpublished observations).

identified by autoradiography at −70°C and purified by three rounds of hybridization as described above.

Sequencing of cDNA Inserts. cDNA inserts from purified phage clones were ligated into pBluescript II SK(−) (Stratagene) or pGEM-7f+ (Promega) by use of appropriate restriction endonucleases (Stratagene, Promega, or United States Biochemical) according to common procedures (13, 14). Dideoxynucleotide sequencing of the cDNA clone was performed with Sequenase II (United States Biochemical) using synthetic oligonucleotide primers made on a Pharmacia Gene Assembler. Sequence comparisons were done using MACVECTOR version 3.5 (International Biotechnologies) and the programs from the University of Wisconsin Genetics Computer Group, version 7 (15).

RESULTS AND DISCUSSION

pCS4, the initial cDNA clone identified by polyclonal antibodies from an expression library, contained only 230 of the 1650 bases predicted for a full-length message (5). Three types of evidence indicated that pCS4 contained CS coding sequences—the observed binding of CS polyclonal antibodies to the fusion protein expressed from pCS4 sequences ligated in a λgt11 library, and the results of both hybrid-arrested and hybrid-selected *in vitro* translation of RNA that annealed to this clone after extraction from elicited seedlings (5). Attempts to isolate any longer clones from the original λgt11 library were never successful, probably because the 230-bp clone was propagated preferentially during library amplification. We suspect that the truncated pCS4 clone arose after incomplete *Eco*RI methylase protection of synthesized double-stranded cDNA was followed by subsequent cloning steps into *Eco*RI-digested λgt11 vector. To facilitate the isolation of a full-length CS cDNA clone, a new library was constructed from a cDNA fraction that was enriched by size fractionation. Also, insertion of the cDNA into the vector was accomplished by the use of adapter molecules rather than linkers. One hundred nanograms of double-stranded cDNA resulted in the production of a primary library containing 550,000 recombinant phage. The initial screen examined 120,000 unamplified phage. Nitrocellulose filter replicates probed with 3′-end pCS4 sequences and with 5′-end genomic sequences containing the transcription start site (unpublished data) identified 35 putative clones that hybridized to both probes. Subsequently, 3 independent recombinant phage clones were purified. Phage clone 28-1 released the largest cDNA insert, about 2 kb, when cleaved with *Not* I restriction endonuclease. The 2-kb *Not* I fragment of clone 28-1 was subcloned to yield pCS7.

pCS7 was sequenced on both strands by the dideoxynucleotide chain-termination method (16). The cDNA insert contained 1983 bp (including a polyadenylate tail of 19 bases) (Fig. 1). The entire pCS4 sequence used as a hybridization probe is found in pCS7 from base 1271 to base 1495. Primer extension experiments using poly(A)$^+$ RNA in conjunction with sequence data from a genomic clone (unpublished observations) showed that the cDNA insert lacked an additional 30 untranslated bases from the 5′ terminus. Translation of the nucleotide sequence revealed one open reading frame that could encode a polypeptide of 601 aa with a molecular weight of 68,960. A putative translation start codon and a possible polyadenylylation signal were also identified.

The minimum transcript needed to encode the *in vitro* translated protein [apparent M_r of 59,000 reported by Moesta and West (4)] would require about 1600 bases. Lois and West (5) had estimated a minimum transcript size of 1650 bases. pCS7 is longer than the predicted minimum size by about 400 bases, which would be of reasonable size to encode the previously purified polypeptide and provide for other expected features.

We have listed the open reading frame beginning at the first codon for methionine rather than the second methionine codon at the eighth residue. The nucleotide sequence flanking the first methionine codon matches 7 of the 12 bases in the consensus for translation start sites in plants (17), whereas the sequences flanking the potential downstream start site are identical only at 4 of the 12 bases. As a result, we favor the first methionine as the putative translation start site.

The FASTA (18) and BLAST (19) algorithms were used to search the GenBank data base (version 63) for similarity of the CS sequence with other reported sequences. Significant similarity was found with the deduced primary sequence of the tobacco EAS (6). Identity between the primary structures of the two proteins is 42%, while the calculated similarity is 65%, as determined by the BESTFIT program (University of Wisconsin Genetics Computer Group) (Fig. 2). Another significant homology was found with the deduced primary sequence of LS from spearmint (31% identity and 53% similarity) (7). As in the case of EAS (6), no significant identity was found with the fungal terpenoid cyclization enzymes *Penicillium* aristolochene synthase (identity, 17%; similarity, 44%) (20) and *Fusarium* trichodiene synthase (identity, 21%; similarity, 47%) (21). Furthermore, little similarity was found with various prokaryotic and eukaryotic enzymes, such as GG*PP* synthase, F*PP* synthase, squalene synthase, and prephytoene diphosphate synthase, which utilize polyprenyl diphosphates as substrates or generate polyprenyl diphosphates as products (identities, 16–23%; similarities, 43–49%).

The alignment of the primary structure of CS with the deduced amino acid sequence of the cytosolic tobacco EAS (6) (J. Chappell, personal communication) suggests that CS may possess a transit peptide. CS has been localized in the proplastid (2) and should contain sequence information in the amino terminus of the initial translation product to direct it into the correct organelle, whereas EAS is thought to be a cytosolic enzyme. The first 49 aa of CS are not aligned with EAS by the BESTFIT program. Thus, it would be reasonable to assume that this 49-aa stretch at the N terminus of CS includes a transit peptide. It does possess some expected characteristics of transit peptides (22), which are often rich in serine and threonine and have a low abundance of acidic amino acids. The N-terminal 49 aa of CS include 11 serine and threonine residues, but only 2 glutamate and aspartate residues. However, no sequence in the 49-aa stretch closely resembles the consensus cleavage sequence for transit peptides (23), so it is not possible to predict a putative cleavage site from this information. If a precursor form of CS with a transit peptide exists, one would predict that it should be larger than the mature form of the enzyme. However, Moesta and West (4) did not observe any significant difference in migration between the mature CS and the *in vitro* translation product in SDS/polyacrylamide gels (4). The basis for this discrepancy, if it is a discrepancy, is not known.

The striking similarities in primary structures for the three terpenoid cyclization enzymes from higher plants illustrated in Fig. 2 raise the question as to why their structures should be so conserved. As noted previously, CS catalyzes the cyclization of GG*PP* to form the diterpene casbene, while EAS catalyzes the cyclization of F*PP* to the sesquiterpene 5-*epi*-aristolochene, and LS catalyzes the cyclization of G*PP* to the monoterpene limonene. Although the sizes of the

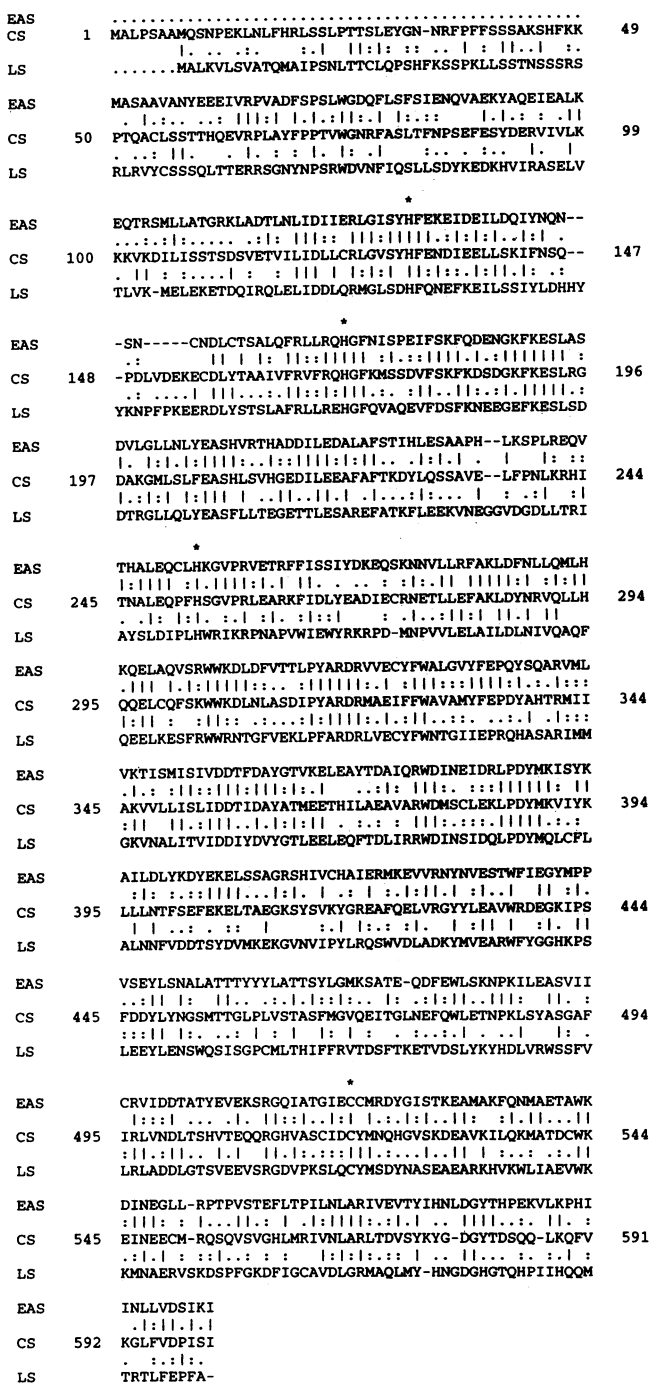

FIG. 2. Comparison of the deduced primary structures of CS from castor bean with those from EAS from tobacco (6) and LS from spearmint (7). Gaps are indicated by dashes. Identities are shown by solid vertical lines. Strong similarities are shown by a colon; weak similarities are indicated by a period. Conserved histidines and cysteine are marked with stars above the alignment. The CS amino acid residue at the end of the line is numbered on the right. Default values for gap penalties for the BESTFIT program were used for this alignment.

prenyl diphosphate substrates differ in the three cases, these enzymes catalyze intramolecular cyclizations of specific prenyl diphosphate to a hydrocarbon via reaction courses that share as a common feature in each case an initial ionization of an allylic diphosphate (24), followed by an attack of the electrons of an internal double bond on the resulting carbocation, and finally, a stabilization of the intermediate by proton abstraction. In addition, LS must also catalyze an

isomerization step converting GPP to (3S)-linalyl diphosphate (25). The reaction courses and intermediates for the three reactions are illustrated in Fig. 3 (26–28). We propose that the similarity in primary structures for these three enzymes reflects the need for common secondary structural features and the positioning of side-chain functional groups that allow for the catalytic conversion of each prenyl diphosphate to its respective hydrocarbon product.

Speculative roles for some of the structural features found in the three enzymes can be suggested on the basis of observations reported in the literature. A putative polyprenyl diphosphate binding site (Fig. 4) has been proposed to coordinate Mg^{2+} or Mn^{2+} cofactors in enzymes that utilize these terpenoid precursors in intramolecular and intermolecular reactions (29). Unspecified aspartic side chains in this binding site were proposed to form direct salt bridges to the metal ion that is also complexed with the diphosphate group. Evidence for this type of interaction between aspartate and Mg^{2+} has been shown in the crystal structure of cyclin-dependent kinase 2 (30), Ha-*ras*-encoded p21 (31), phosphofructokinase (32), and cAMP-dependent protein kinase (33). Other structures show a water molecule hydrogen-bonded between the aspartate and Mg^{2+}—namely, EF-Tu (34), phosphofructokinase (32), and Ha-*ras* p21 (35). Other experimental observations support the idea that an Asp-Asp-Xaa-Xaa-(Asp, Glu) motif contributes to the active site of FPP synthase (36, 37).

Comparison of the deduced primary structures of the three plant enzymes with the consensus putative binding site reveals that only 5 residues in CS are identical with the consensus within the 23-aa stretch (Fig. 4), whereas both EAS and LS have identities with the consensus sequence at 6 positions. However, CS has 10 identities with both EAS and LS within this region (CS residues 347–369). The aspartates are among the conserved residues in all three enzymes,

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EAS   T I S M I S I V D D T F D A Y G T V K E L E A
LS    V N A L I T V I D D I Y D V Y G T L E E L E Q
CS    V V L L I S L I D D T I D A Y A T M E E T H I

Consensus  I G L A F Q I L D D I L D D - G D T A A L G K
           L       L       R   V     S       R
           M
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FIG. 4. Comparison of putative polyprenyl diphosphate binding sites in CS, EAS, and LS with the consensus. Identities shared by all three proteins or between CS and the consensus are shown by vertical lines. Identities shared only by CS and EAS are indicated by broken lines.

suggesting that these side chains are important. Further studies involving the mutation of these residues or active-site affinity labeling should help clarify their possible role in catalysis.

Chemical modification of the active site of LS has suggested important roles for histidine and cysteine residues in its catalytic activity (25, 38). Derivatization of LS *in vitro* with reagents that specifically modify cysteine or histidine residues severely decreased its catalytic activity, whereas preincubation of active enzyme with a nonhydrolyzable transition-state analogue or with Mn^{2+} and GPP protected LS against derivitization by these reagents and the loss of catalytic activity. The alignment of CS, EAS, and LS determined by the PILEUP program in Fig. 2 shows the positions of a single conserved cysteine (CS residue 519) and three conserved histidines (CS residues 131, 171, and 253) in the three proteins. Histidine and cysteine are known to interact with metal ions—e.g., zinc fingers commonly found in transcription factors (39). Perhaps they are also involved in the active sites of CS, EAS, and LS. Although no function is ascribed to these particular residues as yet, they would be

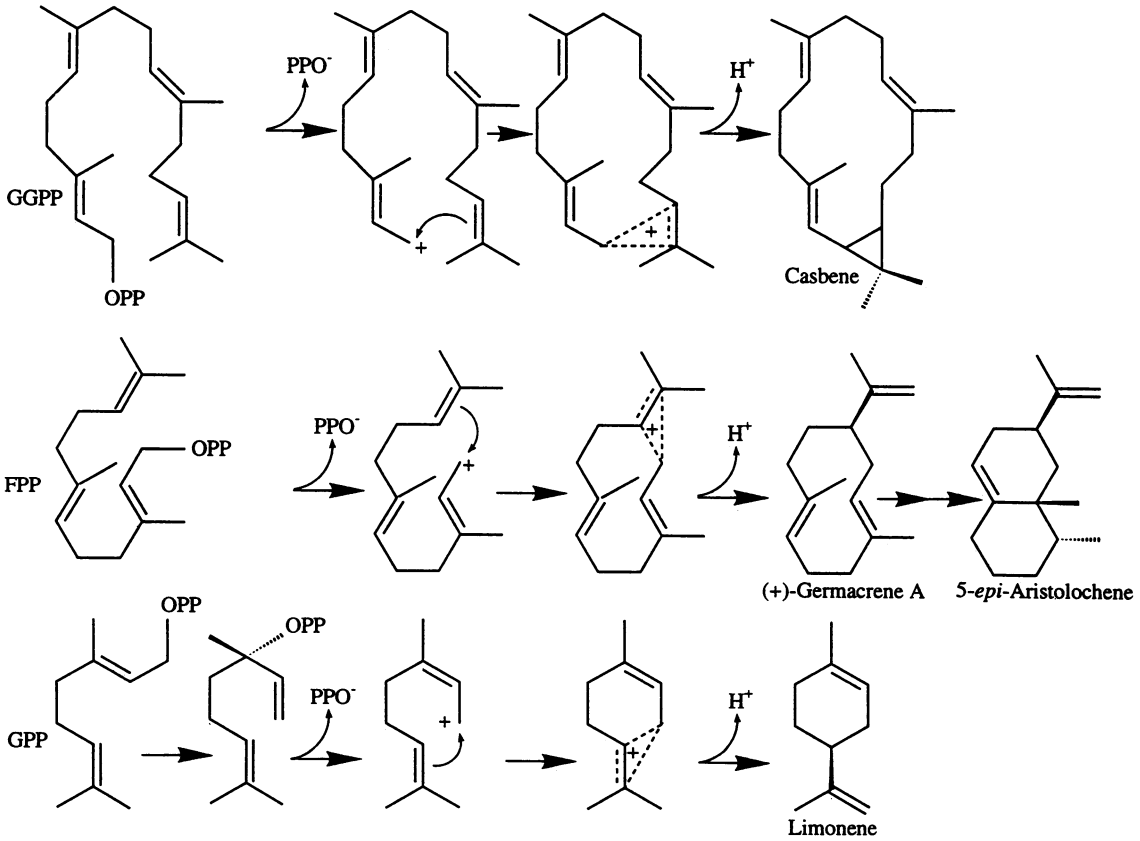

FIG. 3. Comparison of the proposed reaction mechanisms for formation of casbene by CS (26), of 5-*epi*-aristolochene by EAS (27), and of limonene by LS (28).

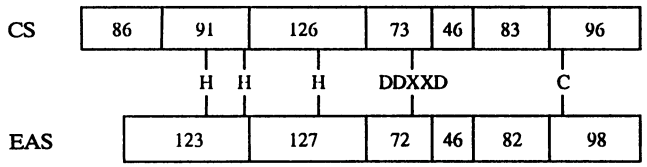

FIG. 5. Comparison of the intron locations in the genes encoding CS and EAS. Vertical solid lines represent the position of the introns within the mRNA for each protein. The positions of conserved amino acid residues are indicated below CS and above EAS by the vertical lines. The number of amino acids encoded within each exon is indicated.

attractive targets for site-directed mutagenesis in structure–function studies.

Predictive secondary structure analysis and hydropathy profiles were utilized to search for possible similarities in the conformational features of CS, EAS, and LS (data not shown). Among other features predicted for each of the enzymes were three similarly positioned conservative stretches of α-helix and a strongly hydrophobic region just N-terminal to conserved aspartates. Secondary structural features determined by conserved regions in the three enzymes could contribute to the positioning of functional groups in the active sites necessary for binding and catalysis.

The three enzymes involved in this comparison were isolated from members of three different plant families—Euphorbiaceae (CS), Solanaceae (EAS), and Lamiaceae (LS). That these enzymes are so similar in much of their primary structures in spite of the taxonomic differences in the species from which they were derived suggests that they arose by divergent evolution from an ancestral gene prior to angiosperm speciation. The correspondence of the positions of five introns in the genes for CS (unpublished observations) and EAS (6) lends further support for this idea. The five introns are nearly identical with respect to the aligned exon coding sequences in the two genes (Fig. 5). This suggests that the exons encode functional domains in the two enzymes that have been highly conserved through evolution. Nothing has been reported to date concerning intron locations in the LS gene. At the same time, it is striking that the fungal enzymes aristolochene synthase and trichodiene synthase, which catalyze cyclization reactions of *FPP* that are mechanistically very similar to the reaction catalyzed by EAS, do not show much primary structural similarity to EAS (6). This low level of primary structural similarity between the plant and fungal enzymes suggests the possibility of convergent evolution in this instance, but more examples and a more careful analysis are needed to evaluate this suggestion.

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