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cDNA Isolation and Characterization of (+)-Germacrene A Synthase from *Ixeris dentata* form. *albiflora* Hara

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***Ixeris dentata* form. *albiflora* Hara, a perennial medicinal herb, accumulates various sesquiterpenes whose first committed step in biosynthesis is the cyclization of farnesyl diphosphate by terpene synthase. To isolate the synthase gene *IdGAS*, we amplified a 184-bp DNA fragment of sesquiterpene synthase gene from *I. dentata* genomic DNA, using a homology-based PCR technique. Sequence information derived from the rapid amplification of cDNA ends was used to produce a 1956-bp full-length cDNA sequence. This included a 1755-b open reading frame for 584 amino acids, with a deduced size of 67.1 kDa and a pI of 5.16. The partially purified recombinant synthase had an optimum temperature and pH at 37°C and 7.5 to 8.0, respectively, as well as a K_m of 11.0 and 14.9 mM at 25 and 37°C, respectively. The expressed protein was inactive with geranyl diphosphate, but did catalyze the cyclization of farnesyl diphosphate to produce a sesquiterpene that was then identified through GC-MS and NMR analyses as (+)-germacrene A. When 44 residues were deleted from its N-terminal, the mutant lost 90% of its activity, suggesting that additional residues are necessary for full enzymatic activity. Transcript levels were comparable between roots and leaves, but began to decline in leaves near the onset of flowering.**

Keywords: Compositae, (+)-germacrene A synthase, *Ixeris dentata*, rapid amplification of cDNA ends, sesquiterpene

Ixeris dentata form. *albiflora* Hara is a perennial medicinal herb indigenous to Korea. This species produces various sesquiterpene guaianolides, such as ixerins (Seto et al., 1986; Chung et al., 1994), which show a wide range of biological activities, including cytotoxic, antifungal, and anti-feedant properties (Ma et al., 1999). For thousands of years, Koreans have consumed this bitter plant as a folk medicine for diabetes and as a springtime delicacy. Guaianolides are well recorded in the members of the Compositae family. For example, *Chrysanthemum boreale*, a wild herbal mum, contains sesquiterpene lactones in its immature flowers, which were suggested to account for its efficacy (Lee et al., 2001).

Sesquiterpene cyclases convert FDP into over 200 different cyclic skeletons. A growing number of these enzymes have been isolated and characterized, including 5-*epi*-aristolochene (Facchini and Chappell, 1992), vetispiradiene (Back and Chappell, 1995), (+)-δ-cadinene (Chen et al., 1995), amorpha-4,11-diene (Chang et al., 2000), *epi*-cedrol (Mercke et al., 1999), δ-selinene (Steele et al., 1998), γ-humulene (Steele et al., 1998), germacrene C (Colby et al., 1998), (+)-germacrene A (de Kraker et al., 2002), (*E*)-α-bisabolene (Bohlmann et al., 1998), and acyclic (E)-β-farnesene (Crock et al., 1997). It is noteworthy that these various structures are produced from a common, simple C_{15} precursor by a family of sesquiterpene synthases, which possesses highly similar tertiary structures among members. The putative key step of guaianolide biosynthesis is the cyclization of FDP by a sesquiterpene synthase to produce a guaiane-type ring. Detection of guaiadiene synthase activity, through which FDP is converted directly into the guaiane ring system, however, has not yet been achieved. Instead, this ring is postulated to arise from a germacrene skeleton via oxidation (de Kraker et al., 1998). In addition, the post-cyclization modification of the germacrene system into a guaiane-type ring has been demonstrated (de Kraker et al., 2001).

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Abbreviations: FDP, farnesyl diphosphate; IdGAS, germacrene A synthase of *Ixeris dentata*; *gISC*, genomic sequence of *Ixeris* sesquiterpene cyclase; RACE, rapid amplification of cDNA ends

We cloned the full-length cDNA of a sesquiterpene synthase from *I. dentata*, and subsequently characterized that the cloned gene is coding (+)-germacrene A synthase. In addition, we studied the role of the N-terminal sequence and transcription of the gene in the plant's aerial portions and roots. Here, we describe IdGAS, another (+)-germacrene A synthase from the Compositae family. Research on the site-directed mutagenesis and detailed kinetic parameters of this enzyme were reported earlier (Chang et al., 2005).

MATERIALS AND METHODS

Plant Materials

Leaves of *I. dentata* form. *albiflora* Hara were purchased from a farm in Yangpyeong, Gyeonggi-do, Korea.

Polymerase Chain Reactions

Genomic DNA and total DNA were prepared from leaf tissues as described previously (Chang et al., 2000). PCR amplification was performed with primers F7 and B9, and its 184-bp product was cloned into a pGEM-T Easy Vector (Promega, USA). Complementary strands were sequenced with an ABI PRISM® Bigdye™ terminator v2.0 cycle sequencing kit and a DNA sequencing system (ABI Model 377SL prism, Applied Biosystems, Perkin-Elmer, USA).

Rapid Amplification of cDNA Ends (RACE)

Preparation of the double-strand cDNA for 3′-RACE was described previously (Chang et al., 2000). Nested primers IXSF (5′-GGTTCCTTACATAAGAGATAGAGTACC-3′) and UAP were used for the PCR. Single-strand cDNA for 5′-RACE was obtained with the GeneRacer kit (Invitrogen, USA) and the gene-specific primer IXS3B (5′-CGTCTTGTAGCCTTGAAATCAACTCTG-3′). This cDNA was ligated with a GeneRacer™ RNA oligomer containing the GeneRacer™ nested primer (5′-GGACACTGACATGGACTGAAGGAGTA-3′), according to manufacturer's instructions. Nested primer IXSB (5′-GGTACTCTATCTCTTATGTAAGGAACC-3′) and the GeneRacer™ nested primer were used for the PCR.

Bacterial Expression

Based on the sequences from RACE, a pair of IXSTART (5′-GGATCCATGGCTCTCGTGAGAAAC-3′; *Bam*HI site underlined) and IXSTOP (5′-CTAGCAGCTTTCAAGACCAGGTG-3′) primers was designed to obtain a 1755-bp PCR product, which had the open reading frame (ORF) of sesquiterpene synthase. This product was cloned into a pEZ-T vector (RNA, Korea). The resulting plasmid was digested with *Bam*HI and *Kpn*I, and cloned into a pRSET-a expression vector (Invitrogen). Transformation, enzyme induction, cell extraction, and SDS-PAGE were performed as described (Chang et al., 2000).

Generation of Truncated GAS

A pair of primers, TruncB (5′-TTGGCCAACTTCCCGCCTTC-3′) and TruncF1 (5′-AGCCATGGATCCCCATCGATC-3′), was used to amplify the D44 truncate mutant from pRSET-a, which harbors the full ORF of *IdGAS*. Similarly, the use of TruncF2 (5′-CCGACTCCTGAGCCAGTTAGA-3′) and TruncF3 (5′-CTGGCTGTCCAACCGACTCCT-3′) in combination with TruncF1 yielded D36 and D32, respectively.

Quantitative PCR

Total RNA and cDNA were prepared as described in the PCR and RACE section. Quantitative PCR was performed in triplicate on a Rotor-Gene 2000 (Corbett Research, Australia) with a 20-mL mixture consisting of 10 μL of Quantitect™ CYBR Green PCR master mix (2×), 1 μL each of the forward (GAAGCTTACGCAAATGCACA-3′) and backward (GCGGTGCACTGAGTAGATCA-3′) primers (which represented 119 bp of the germacrene synthase-specific 5′-region), 2 μL cDNA, and 6 μL H_2O. The temperature program included: initiation at 95°C for 15 min; followed by 45 cycles of 94°C for 15 s, 50°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 1 min. Quantification was performed against the standard ORF of GAS as obtained by PCR, and was calculated using Rotor-Gene 2000 Version 4.2 software. The copy number was calculated as described by Bishop et al. (1997).

Preparation of Black Pepper Extract

Forty grams of black pepper powder (Ottogi Co., Korea) was hydro-distilled in a glass apparatus for 5 h to yield 150 μL essential oil (Schultz et al., 1977). The oil was analyzed by an HP5890 Series II gas chromatograph (Hewlett-Packard, Germany) equipped with an AX505WA mass spectrometer (JEOL, Japan)

operating in the electron impact mode. GC-MS conditions included: injector temperature, 250°C; flow rate, 2 mL min^{-1}; N_2 carrier gas; split ratio, 5:1; Cyclosilb column (30 cm × 0.25 mm, 0.25 m); temperature program of 70°C for 1 min, 5°C min^{-1} increased to 210°C, and 210°C for 5 min. Elemene was eluted at 18.68 min (Prosser et al., 2002).

Preparation of Purified Enzyme and Sesquiterpene Cyclase Assay

After the expressed enzyme was partially purified with ProBond™ Nickel-chelating resin (Invitrogen), a 0.14-mg sample was incubated overnight with 27 mM FDP at 25°C in 3.0 ml of the reaction mixture. The product was then extracted with HPLC-grade hexane, filtered through a small alumina column, and concentrated to dryness for spectral analyses. GC-MS was performed as described in the previous section. An NMR spectrum [^{1}H NMR (600 MHz, $CDCl_3$): 5.25 (1H, br s, H-5), 4.65 (1H, br s, H-12a), 4.63 (1H, br d, J=1.5 Hz, H-1), 4.63 (1H, br s, H-12b), 1.77 (6H, s, H-15, H-13), 1.54 (3H, s, H-14)] was obtained with 2000 transients on an Advance 600 (Bruker, Germany), using $CDCl_3$ as a solvent in a 185-μL WILMAD microprobe NMR tube.

Characterization of Sesquiterpene Synthase

To examine its activity, the purified enzyme was diluted ten-fold and incubated at 25°C in a reaction mixture of 30 mM HEPES (pH 7.0), 3.6 mM $MgCl_2$, 3 mM β-mercaptoethanol, 2.6 mM DTT, 0.4 to 32.0 mM FDP that contained 5.55 kBq [1-^{3}H]FDP (0.596 TBq mol^{-1}, NEN, USA), and 2 mM FDP (Sigma, USA). This mixture was then extracted with hexane and filtered as described above. Radioactivity of the filtrate, representing the amount of sesquiterpene product, was determined through scintillation counting. After establishing the linear increase in reaction products for up to 60 min under the given conditions, the optimum pH, temperature, and concentrations of Mg^{2+} and Mn^{2+} were determined based on a 30-min reaction and the K_m value were obtained under the optimal conditions.

RESULTS

Partial Sequence of Terpene Synthases

We used homology-based PCR to amplify a 184-bp-long DNA fragment (data not shown) of the sesquiterpene cyclase gene from genomic DNA of *I. dentata* leaves. As predicted, the size of the product suggested that the intron was absent between primer sites (Steele et al., 1998). This major PCR product was cloned into a pGEM-T vector, and two partial genomic clones (Fig. 1) of *Ixeris* sesquiterpene cyclases were obtained--*gISC1* and *gISC2* (sequence readings from 11 and 5 colonies, respectively). A comparison of the deduced 44 amino acid sequences of *gISC1* to *gISC2* and to *Nicotiana tabacum* 5-*epi*-aristolochene synthase (TEAS) displayed 38% and 43% identities, respectively, while *gISC2* to TEAS showed 54% identity. Nevertheless, the $R^{266,268}$ and W^{275} in aristolochene synthase, which are suggested to facilitate the ionization of FDP and the stabilization of the resulting carbocation, respectively, were aligned with both genes. In lettuce and chicory, where two isogenes for germacrene A synthases were present, very high amino acid homology (>90%) was found in the 44-residue region among the genes. Therefore, it is unlikely that *gISC1* and *gISC2*, with only 38% homology, have the same catalytic function.

Identification of a Full-length cDNA

Based on the partial genomic sesquiterpene synthase sequence of *gISC1*, sequences for 1036- and 947-bp products were obtained from 3'- and 5'-RACEs (readings from 8 and 6 colonies), respectively. Combining that RACE sequence information yielded a full-length cDNA sequence of 1956 b (GenBank/EMBL accession No. AY082672). With a start codon

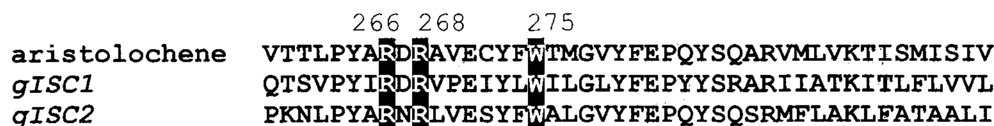

Figure 1. Alignment of the deduced amino acid sequences of *gISC1* and *gISC2*. $R^{266,268}$ and W^{275} are highly conserved catalytic amino acid residues involved in carbocation stabilization and hydrophobic interaction of helical loop over active site, respectively.

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           ▼2                                  ▼32 ▼36   44▼
IdGAS      MALVRNNSINGREPVLSPRSLTSPRGLTSPRPLAVQPTPEPVRPLANFPPSIWANRFISFSLDNSE-LEA 69
GASlo      MALVRNNSSNGREPVLSPRSLTSPRGLTSPRPLSVQPTPEPVRPLANFPPSIWADRFISFSLDNSQ-LEA 69
LTC1       MAAVEAN---------------------GTLQANTKTTTEPVRPLANFPPSVWGDRFLSFSLDNTE-LEG 48
GASsh      MAAVEAN---------------------GTFQANTKTT-EPVRPLANFPPSVWGDRFLSFSLDTTE-LEG 47
LTC2       MAAVDTN---------------------ATIQE--KTTAEPVRPLANFPPSVWGDRFLSFTLDNSE-LEG 46
Sc1        MAA-----------------------------KQ-VEVIRPVANYHPSLWGDQFLHY--DEQEDEHV 35
TEAS       MAS---------------------------AAVANYEEEIVRPVADFSPSLWGDQFLSFSIDNQVAEKY 42

IdGAS      YANAHEEPKESVRSLITDTTID----ASAKLKLIYSVHRLGLSYLYPEEIDAELDQLFKKIDLHHYEQVD 135
GASlo      YANALEEPKEAVKSLITDTTID----ANTKLKLIYSVHRLGLSYLYPDEIDAELNKLFEKIDLQYYEQVD 135
LTC1       YAKAMEEPKEEVRRLIVDPTMD----SNKKLSLIYSVHRLGLTYLFLQEIEAQLDNIFKAFKLQDYDEVD 114
GASsh      YAKAMEEPKEEVRKLIVDPTMD----SNKKLSLIYSVHRLGLTYLFLQEIEAQLDKLFKEFNLQDYDEFD 113
LTC2       YAKAMEAPKEELRRLIVDQTMD----SNKKLSLIYSVHRLGLTYLFLQEIEAQLDKIFKEFNLQNYDEVD 112
Sc1        EVDQQIEILKEETRKEILASLDDPTKHTNLLKLIDVIQRLGIAYYFEHEITQALDHIYSVYGDEWNGGR- 104
TEAS       IYAQEIEALKEQTRSMLLATGRKLADTLNLIDIIERLGISYHFEKEIDEILDQIYNQNSNCN----DLCT 108

IdGAS      LYTISVQFQVFRHHGYKLSSDIFKKFKDTTTGTFTDEVTKDVKGMLSLYESAHLRLHGEEILDEALVFTE 205
GASlo      LYTIAVQFQVFRHHGYKISSDVFKKFKDSTTGTFTDDVTKDVKGMLSLYESAHLRLHGEDILDEALAFTE 205
LTC1       LYTTSINFQVFRHLGHKLPCDVFNKFKDSSSGTFKESITNDVKGMLGLYECAQLRLRGESILDEASAFTV 184
GASsh      LYTTSINFQVFRHLGHKLPCDVFNKFKDSSSGTFKESITNDVKGMLGLYESAQLRLRGEPILDEASAFTE 183
LTC2       LYTTSINFQVFRHLGYKLPCDVFNKFKDNTSGAFKEDISTDVKGMLGLYESSQLRTRGESILDEASSFTE 182
Sc1        ---TSLWFRLLRQQGFYVSCDIFNIYKLD-NGSFKDSLTKDIECMLELYEAAYMRVQGEIILDEALEFTK 170
TEAS       SALQFRLLRQHGFNISPEIFSKFQDEN-GKFKESLASDVLGLLNLYEASHVRTHADDILEDALAFSTIHL 177

IdGAS      AQLKKIVSTLEG---DLANQVNQVLKRPFHTGMPMVEARLYFNTHEEDVSCYESIVKLAKVHFNYLQLQQ 272
GASlo      AHLKKILTTLEG---DLARQVNQVLKRPFHTGMPMVEARLYFITHEEDFSSHESVVKLAKVHFNYLQLQQ 272
LTC1       TQLKSVVNTLEG---KLAQQVLQSLKRPFHQGMPMVEARFYFSNYDEECSTHESLVKLAKLHFNYLQLQQ 251
GASsh      TQLKSVVNTLEG---NLAKQVMQSLRRPFHQGMPMVEARMYFSNYDEECSTHESLPKLAKLHFNYLQLQQ 250
LTC2       TKLKSVVNNLEG---NLAQQVLQSLRRPFHQGMPMVEARLYFSNYSEECATHECLLKLAKLHFSYLELQQ 249
Sc1        THLEHIAKDPLRCNNTLSRHIHEALERPVQKRLPRLDAIRYIPFYEQQDSHNKSLLRLAKLGFNRLQSLH 240
TEAS       ---ESAAPHLKSPLREQVTHALEQCLHKGVPRVETRFFISSIYDKEQ--SKNNVLLRFAKLDFNLLQMLH 242

               F7→          296 298    305                    333,4 337 ←B9
IdGAS      KEELRIVSQWWKDMEFQTSVPYIRDRVPEIYLWILGLYFEPYYSRARIIATKITLFLVVLDDTYDAYATI 342
GASlo      KEELRLVSQWWKDMQFQQSVPYIRDRVPEIYLWILGLYFEPYYSRARIIATKITLFLVVLDDTYDAYATI 342
LTC1       KEELRIVSKWWKDMRFQETTPYIRDRVPEIYLWILGLYFEPRYSLARIIATKITLFLVVLDDTYDAYATI 321
GASsh      KEELRIVSKWWKDMRFQETTPYIRDRVPEIYLWILGLYFEPRYSLARIIATKITLFLVVLDDTYDAYATI 320
LTC2       KEELRIVSKWWKDMRFQETTPYIRDRVPEIYLWILGLYFEPRYSLARIIATKITLFLVVLDDTYDAYATI 319
Sc1        KKELSQLSKWWKEFDAPKNLPYVRDRLVELYFWILGVYFEPQYSRSRIFLTKTIKMAAILDDTYDIYGTY 310
TEAS       KQELAQVSRWWKDLDFVTTLPYARDRVVECYFWALGVYFEPQYSQARVMLVKTISMISIVDDTFDAYGTV 314

IdGAS      DEIRAITDAINRWEMSAIDLLPEYIKPFYRILLNEYDDLEKEYSKDGRAFSVHASKQAFQEIARGYLEEA 412
GASlo      DEIRSITDAINRWEISAIDQLPEYIKPFYRILLNEYDDLEKEYSKDGRAFSVHASKQAFQEIARGYLEEA 412
LTC1       EEIRLLTDAINRWDISAIEQIPEYIRPFYKILLDEYAELEKQLAKEGRAKSVIALKEAFQDIARGYLEEA 391
GASsh      EEIRLLTDAINRWDISAMEQIPEYIRPFYKILLDEYAELEKQLAKEGRAKSVIASKEAFQDIARGYLEEA 390
LTC2       EEIRLLTDAINRWDMSAMEQIPEYIRPFYKILLDEYAELEKQLAIEGRAKSVIASKEAFQDIARGYLEEA 389
Sc1        EELEIFTKAVQRWSITCMDTLPDYMKVIYKSLLDVYEEMEEIIEKDGKAYQVHYAKESMIDLVTSYMTEA 380
TEAS       KELEAYTDAIQRWDINEIDRLPDYMKISYKAILDLYKDYEKELSSAGRSHIVCHAIERMKEVVRNYNVES 384

                                                                    474 478
IdGAS      EWLHNGYVATFPEYMKNGLITSAYNVISKSALVGMG-AIADEEALAWYETHPKILKASELISRLQDDVMT 481
GASlo      EWLHNGYVATFPEYMKNGLITSAYNVISKSALVGMG-AIADEEALAWYETHPKILKASELISRLQDDVMT 481
LTC1       EWTNSGYVASFPEYMKNGLVTSAYNVISKSALVGMG-EMVSEDALAWYESHPKTLQASELISRLQDDVMT 461
GASsh      EWTNSGYVASFPEYMKNGLITSAYNVISKSALVGMG-EMVGEDALAWYESHPKTLQASELISRLQDDVMT 459
LTC2       EWTNSGYVASFPEYMKNGLITSAYNVISKSALVGMG-DIVSENALAWYESHPKTLQASELISRLQDDVMT 458
Sc1        KWLHEGHVPTFDEHNSVTNITGGYKMLTASSFVGMHGDIVTQESFKWVLNNPPLIKASSDISRIMNDIVG 450
TEAS       TWFIEGYMPPVSEYLSNALATTTYYYLATTSYLGMK--SATEQDFEWLSKNPKILEASVIICRVIDDTAT 452

IdGAS      FQFERKRGQSATGVDAYIKEYNVSEEVAIKDLMKMIENAWKDINEGCLKPTEVSVALLTPILNLARMIDV 551
GASlo      FQFERKRGQSATGVDAYIKEYNVSEEVAIKELMKMIENAWKDINEGCLKPTEVSVALLTPILNLARMIDV 551
LTC1       YQFERERGQSATGVDSYIKTYGVSEKEAIDELNKMIENAWKDINEGCLKPREVSMDLLAPILNLARMIDV 530
GASsh      YQFERERGQSATGVDSYIKTYGVTEKEAIDELNKMIENAWKDINEGCLKPREVSMDLLAPILNLARMIDV 529
LTC2       YQFERERGQSATGVDAYIKTYGVSEKEAIDELKIMIENAWKDINEGCLKPREVSMDLLAPILNLARMIDV 528
Sc1        HKEEQQRKHIASSVEMYMKEYNLAEEDVYDFLKERVEDAWKDINRETLTCKDIHMALKMPPINLARVMDM 520
TEAS       YEVEKSRGQIATGIECCMRDYGISTKEAMAKFQNMAETAWKDINEGLLRPTPVSTEFLTPILNLARIVEV 522

           553 557 559
IdGAS      VYKFD-DGFTFPGKTLKDYITLLFVTPAPSLESC 584
GASlo      VYKFD-DGFTFPGKTLKDYITLLFVSPPPSLEN- 583
LTC1       VYRYD-DGFTFPGKTMKEYITLLFVGSSPM---- 559
GASsh      VYRYD-DGFTFPGKTMKEYITLLFVGSSPM---- 558
LTC2       VYRYD-DGFTFPGKTMKEYITLLFVGSVSM---- 557
Sc1        LYKNG-DNLKNVGQEIQDYMKSCFINPMSV---- 549
TEAS       TYIHNLDGYTHPEKVLKPHIINLLVDSIKI---- 550
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Figure 2. Amino acid sequence alignment of *IdGAS* (GenBank accession no. AY082672) with related plant (+)-germacrene A synthases from chicory (*GASlo*, AF497999; *GASsh*, AF498000), lettuce (*LTC1*, AF489964; *LTC2*, AF489965), and goldenrod (*Sc1*, AJ304452). *TEAS* is a tobacco *epi*-aristolochene synthase (L04680). Characteristic amino acid residues of plant sesquiterpene synthase, involved in catalytic triad and other active sites (Starks et al., 1997), are labeled with residue numbers and shown in gray boxes. Solid triangles denote cut positions for N-terminal deletion mutants. Regions for PCR primer pairs, F7 and B9, are underlined with forward and backward arrows, respectively, for polymerization direction. Alignment was made using ClustalW software.

ATG and a stop codon TAG at 49 and 1801, respectively, in frame with the ORF, we called this *IdGAS*. A typical poly(A^+) signal (AATAAT/A) occurred twice between positions 1917 and 1922. The deduced 584-amino acid sequence of the 1755-bp ORF showed a calculated MW and pI of 67.1 kDa and 5.16, respectively.

When we compared this newly deduced amino acid sequence with those of short and long (+)-germacrene A synthase-encoding genes from chicory (Bouwmeester et al., 2002) as well as with another pair of (+)-germacrene A synthase-encoding genes (*LTC1* and *LTC2*) from lettuce (Bennett et al., 2002), we found 71% and 92%, and 71% and 72% identities, respectively (Fig. 2). In contrast to the high homology seen with germacrene A synthases, IdGAS shared only 41% and 36% identities with TEAS (GeneBank Accession L04680) and germacrene C synthase (AF035630), respectively.

Bacterial Expression and Characterization

To determine the function of IdGAS, the ORF was cloned into the pRSET-a expression vector with a pair of IXSTART and IXSTOP primers. SDS-PAGE analysis revealed an 67-kDa protein in the insoluble and partially purified soluble fractions of transformed *Escherichia coli* (Fig. 3). Purity of the enzyme preparation

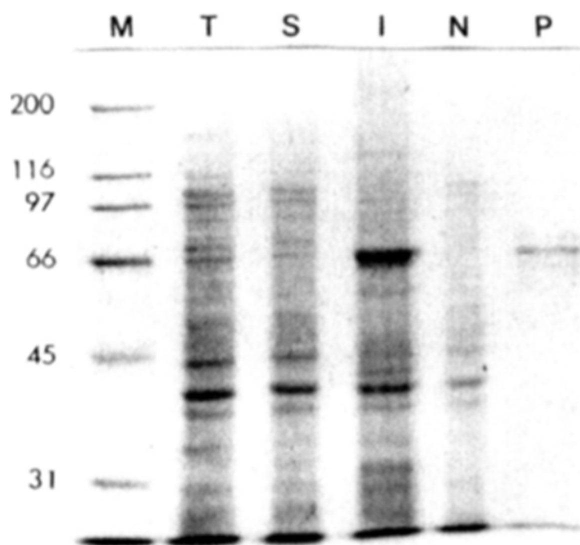

Figure 3. Electropherogram of expressed *IdGAS* (arrow). M, molecular weight marker; T, total extract of transformed *E. coli*; S, soluble fraction; I, insoluble fraction; N, soluble fraction from *E. coli* harboring plasmid only; P, Ni column-purified fraction. Numbers indicate values in KDa.

was not further established because it was irrelevant to the present preliminary kinetic study. The enzymatic reaction of the soluble fraction from *E. coli* with FDP as substrate produced a hydrocarbon product against the background of an empty vector transformant. In addition, the expressed protein IdGAS, being inactive against geranyl diphosphate, was devoid of monoterpene synthase activity. The total ion chromatogram from GC-MS analysis of the hydrocarbon fraction revealed a peak with a retention time at 18.66 min and a molecular ion at *m/z* 204 (Fig. 4). Selective ion monitoring of the reaction product at *m/z* 204 showed a single peak with the same retention time. A library search indicated this fragmentation pattern is compatible with that of β-elemene rearranged from germacrene A (Weinheimer et al., 1970). The retention times and the fragmentation patterns of the product and (–)-β-elemene in black pepper oil, as confirmed by co-injection, were identical (data not shown), an indication that the enzymatic product is (+)-germacrene A (Prosser et al., 2002).

Further confirmation of the product structure was obtained from a ^1H NMR spectrum of freshly prepared samples from a large-scale experiment using 50 mg FDP. It presented the two terminal vinyl protons (H-12a and -12b) and two vinyl protons (H-1 and -5) expected from the germacrene A skeleton. Three allyl methyl groups (H-13, -14, and -15) were also evident. This assignment was based on the report of Weinheimer et al. (1970) and through the aid of ACD/Structure Elucidator Version 4.5 (Advanced Chemistry Development, Canada). After standing for several days at room temperature, the aged sample exhibited spurious peaks, presumably due to rearrangement.

The partially purified recombinant synthase showed maximum activity at a pH of 7.5 to 8.0, 37°C, and 5 mM Mg^{2+}. However, at the same concentration of Mn^{2+}, maximum activity was only about one-fifth (Fig. 5), while at a higher Mg^{2+} concentration (i.e., 100 mM), activity was significantly reduced. We calculated the K_m value for FDP with the recombinant synthase to be 11.0 μM at 25°C, a value typical for other sesquiterpene synthases of plant origin (Chang et al., 2005). However, this K_m value was somewhat higher at 37°C, reaching 14.9 μM.

Role of N-Terminal Sequence

Compared with other germacrene A synthases, IdGAS had approximately 20 additional amino acid residues that were rich in S and T, with the exception

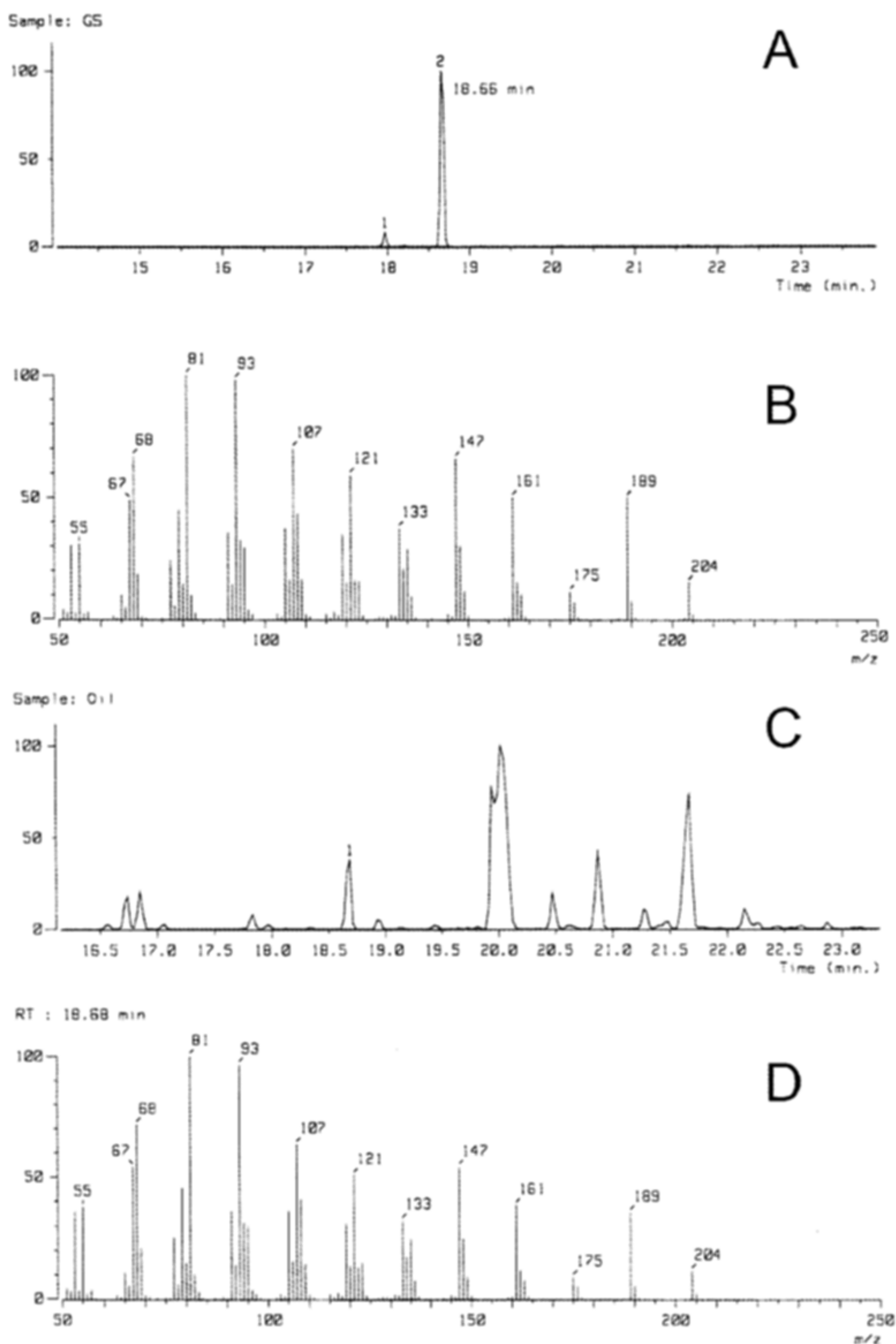

Figure 4. GC-MS analysis of reaction product of (+)-germacrene synthase on Cyclosilb chiral column. **(A)** Total ion chromatogram of enzyme reaction product using FDP as substrate. **(B)** Mass spectrum of synthase reaction product derived from FDP (R_t = 18.66 min). Identity of small peak at 17.9 min is unknown. Temperature of GC analysis allowed rearrangement of enzymatic product into (-)-β-elemene. **(C)** Total ion chromatogram of black pepper oil. **(D)** Mass spectrum of (-)-β-elemene from black pepper oil eluted at 18.68 min.

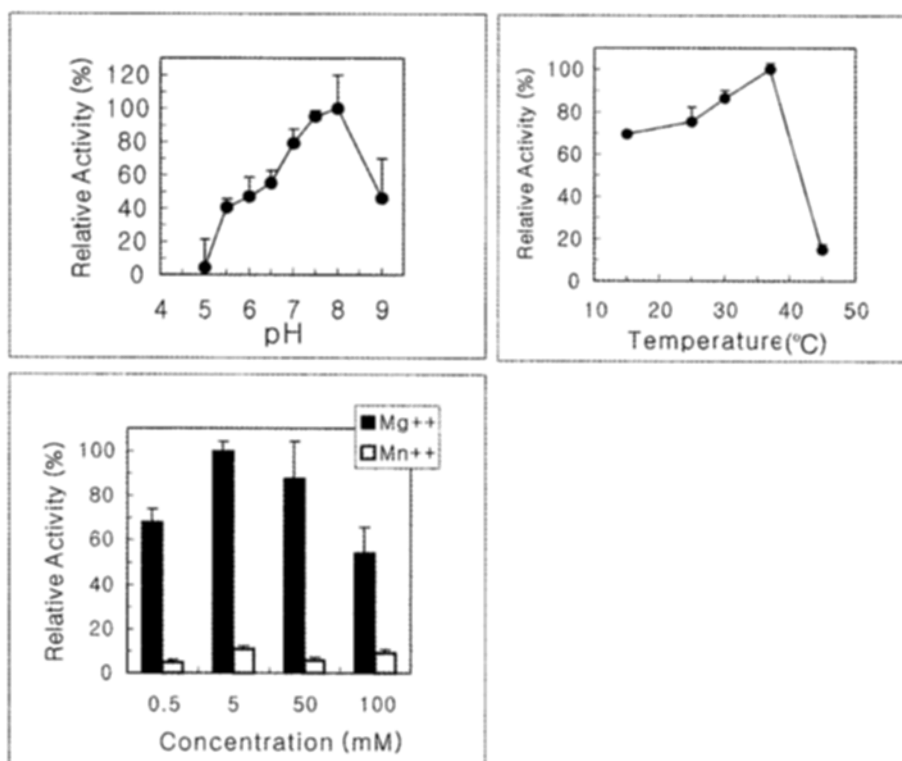

Figure 5. Determination of optimal pH, temperature, and metal ion requirement for (+)-germacrene A synthase (clockwise from upper left box).

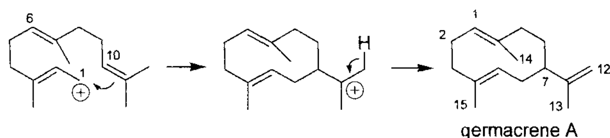

Figure 6. Proposed cyclization mechanism of germacrene A from farnesyl diphosphate.

of GASlo (Fig. 2). Crystallographic study of TEAS has demonstrated that a disordered N-terminal region, V^{17} to N^{36}, can become ordered upon the binding of substrate (Starks et al., 1997). Therefore, to determine if the additional sequence was necessary for enzyme activity, we prepared three truncated IdGAS mutants --Δ44, Δ36, and Δ32, to comprise the corresponding disordered region in TEAS (Fig. 2). These mutants were purified, along with the wild-type enzyme, on a Ni column. When assayed under the conditions described earlier, they lost about 90% of their relative activity compared with the wild type. These results imply that the S- and T-rich N-terminal region was not a transit peptide. The shorter N-terminal regions in other germacrene synthases (Fig. 2) suggested that the additional N-terminal sequence may be a nonessential part of the cyclase. However, the present study suggests that the extra sequence, being found so far only in GASlo of chicory and IdGAS, may play a role in the cyclization of germacrene A.

Transcription of IdGAS in *I. dentata*

Quantitative PCR analyses showed that the level of IdGAS transcription fluctuated during the growing season. For example, plants sampled earlier in the year, (i.e., on April 16) exhibited about equal transcriptional activity between the leaves (36,600 ± 3,300 copies μg^{-1} RNA) and the roots (34,400 ± 3,900 copies μg^{-1}). However, when the plants reached the flowering stage (May 18), a significant shift was observed toward the roots (75,500 ± 7,800 copies μg^{-1}) versus the leaves (only 7,100 ± 1,400 copies μg^{-1}).

DISCUSSION

We have now isolated two partial clones of putative sesquiterpene synthase genes *gISC1* and *gISC2* from the genomic DNA of *I. dentata*, and have cloned,

from its cDNA pool, one full open reading frame that contains the sequence for *gISC1*. This newly cloned gene has been functionally identified as *IdGAS*. It is unclear whether guaianolide arises from the direct cyclization of farnesyl diphosphate or via multi-step transformation of (+)-germacrene A (Fig. 6). No one has yet reported a cloning of a terpene cyclase that catalyzes a second cyclization leading to the guaiadiene system. A series of enzymatic activity has been described for the biosynthetic pathway of guaianolides, including (+)-germacrene A synthase, (+)-germacrene A hydroxylase, and NADPH-dependent sesquiterpenoid dehydrogenase, in the roots of chicory, a species with high concentrations of guaianolides (de Kraker et al., 1998, 2001). Incubating germacratrienoic acid with the microsomal fraction of chicory roots produces guaianolide as well as costunolide (de Kraker et al., 2002), thereby favoring a scenario of post-cyclization modification.

Two (+)-germacrene A synthase-encoding genes, *GASsh* and *GASlo*, have been cloned and characterized from chicory (Bouwmeester et al., 2002). The abundant occurrence of guaiadienes in plant sources, such as patchouli oil, suggests alternative, direct formation of the hydrocarbon by cyclase(s) (Munck and Croteau, 1990), while prior formation of a lactone moiety before closure of the second ring may be operative in the Compositae family (de Kraker et al., 2002). The failure to clone a hypothetical guaiadiene synthase gene in Compositae would strongly indicate a post-cyclization scenario. Indeed, the presence of such enzyme patchoulol synthase has been described in *Pogostemon cablin* (Munck and Croteau, 1990). It would be very interesting to determine why two separate sesquiterpene cyclization systems exist in nature, i.e., one directly forming two-ring systems (such as aristolochene and amorphadiene); the other mobilizing post-cyclization modification from germacrene to arrive at certain other two-ring systems (e.g., guaianolide and costunolide).

Although their exact role in plant physiology is unknown, some guaianolides might be involved in complex chemical ecology (Harborne, 1999). For example, lactucin in chicory and parthenin in *Parthenium hysterophorus* may act as anti-feedants and toxins for herbivorous insects. In support of this theory, we observed fluctuations in the leaf and root transcription patterns of *IdGAS* during the life cycle of *I. dentata*. Here, during the short period of vegetative growth (April through early May), the gene was equally active in both tissue types. Transcription at that time would ultimately lead to the biosynthesis of guaianolide. However, transcript levels were ten times greater in the root than in the leaves when plants began to bloom in mid-May. This change might have signified that guaianolide was being preferentially stored in the roots just before the aerial portions were about to perish after flowering. Such seasonal variation of sesquiterpene content has been also noted in lacquer of *Dendropanax morbifera* (Ahn et al., 2003). Therefore, these seasonal fluctuations and altered distribution of transcription level of *IdGAS* among plant tissues may serve a protective role against attack by herbivorous insects.

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