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The Arabidopsis *GA1* Locus Encodes the Cyclase *ent*-Kaurene Synthetase A of Gibberellin Biosynthesis

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The first committed step in the gibberellin (GA) biosynthetic pathway is the conversion of geranylgeranyl pyrophosphate (GGPP) through copalyl pyrophosphate (CPP) to *ent*-kaurene catalyzed by *ent*-kaurene synthetases A and B. The *ga1* mutants of Arabidopsis are gibberellin-responsive male-sterile dwarfs. Biochemical studies indicate that biosynthesis of GAs in the *ga1* mutants is blocked prior to the synthesis of *ent*-kaurene. The *GA1* locus was cloned previously using the technique of genomic subtraction. Here, we report the isolation of a nearly full-length *GA1* cDNA clone from wild-type Arabidopsis. This cDNA clone encodes an active protein and is able to complement the dwarf phenotype in *ga1-3* mutants by Agrobacterium-mediated transformation. In *Escherichia coli* cells that express both the Arabidopsis *GA1* gene and the *Erwinia uredovora* gene encoding GGPP synthase, CPP was accumulated. This result indicates that the *GA1* gene encodes the enzyme *ent*-kaurene synthetase A, which catalyzes the conversion of GGPP to CPP. Subcellular localization of the GA1 protein was studied using ^{35}S-labeled GA1 protein and isolated pea chloroplasts. The results showed that the GA1 protein is imported into and processed in pea chloroplasts in vitro.

INTRODUCTION

Gibberellins (GAs) are a large family of diterpenoid compounds, some of which are bioactive growth regulators, controlling such diverse processes as germination, cell elongation and division, and flower and fruit development. Extensive biochemical studies on endogenous GA intermediates in GA-responsive dwarf mutants have allowed the determination of the GA biosynthetic pathway and several genetic loci involved in GA biosynthesis (reviewed by Graebe, 1987). The synthesis of geranylgeranyl pyrophosphate (GGPP) from mevalonate is common to terpenes. GGPP is a branch point metabolite that is not only the precursor of GAs but also a precursor of other diterpenes, such as the phytol chain of chlorophylls, and tetraterpenes, such as the carotenoids. The first committed step of the GA pathway is the conversion of GGPP to *ent*-kaurene in a two-step cyclization reaction. GGPP is partially cyclized to the intermediate copalyl pyrophosphate (CPP) by *ent*-kaurene synthetase A, and CPP is immediately converted to *ent*-kaurene by *ent*-kaurene synthetase B. Because *ent*-kaurene is a key intermediate in the GA pathway, its synthesis is likely to be a regulatory point for GA biosynthesis. Indeed, *ent*-kaurene production has been shown to be altered by changes in photoperiod, temperature, and growth potential of tissues in certain species (Chung and Coolbaugh, 1986; Moore and Moore, 1991; Zeevaart and Gage, 1993).

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Arabidopsis *ga1* mutants are male-sterile dwarfs whose phenotype can be converted to the wild type by repeated application of GA (Koornneef and van der Veen, 1980). Several of the more severe *ga1* alleles also exhibit a nongerminating phenotype (Koornneef et al., 1983). The *ga1* mutants contain reduced levels of GAs (M. Talon and J.A.D. Zeevaart, personal communication), and the *ent*-kaurene synthetase activity in cell-free preparations from *ga1* mutants is very low compared to the wild type (Barendse and Koornneef, 1982; Barendse et al., 1986). Zeevaart et al. (1986) reported that application of *ent*-kaurene also restored growth of the *ga1* mutants and that ^{14}C-*ent*-kaurene was metabolized to GAs when applied to the leaves of these mutants. These results suggest that GA biosynthesis in the *ga1* mutants is blocked prior to the formation of *ent*-kaurene, but the rest of the pathway is unaffected by the mutation. Because the *ga1* mutants produce chlorophylls and carotenoids, it is unlikely that the mutation affects the synthesis of GGPP. Therefore, the *GA1* locus is probably involved in the conversion of GGPP to *ent*-kaurene, encoding one of the *ent*-kaurene synthetases or a regulator needed for formation of the active enzyme. To study the function of the GA1 protein and the regulation of the *GA1* gene in relation to GA biosynthesis, we isolated the *GA1* locus previously by genomic subtraction (Sun et al., 1992).

In this study, we present the nucleotide sequence of a *GA1* cDNA clone of 2.6 kb containing a 2.4-kb open reading frame (ORF) and the structure of the 7-kb *GA1* locus. Based on the

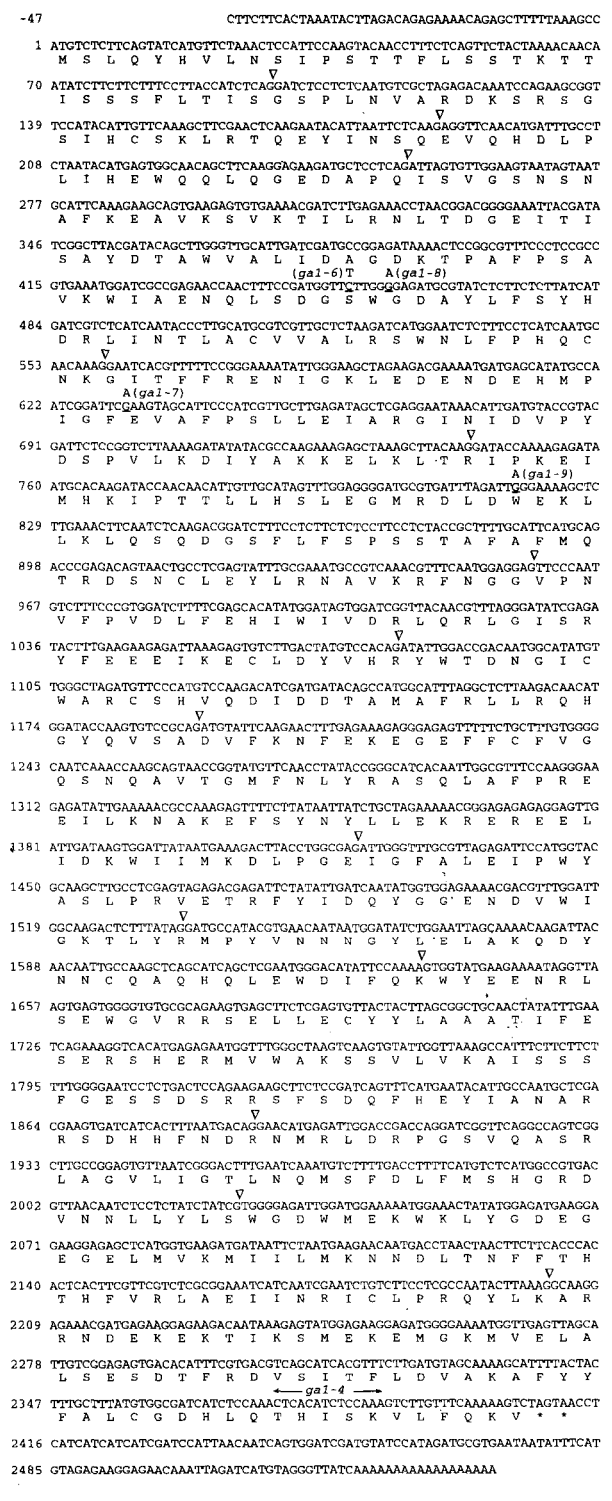

Figure 1. Nucleotide Sequence of 2.6-kb *GA1* cDNA and Predicted Amino Acid Sequence.

The nucleotide sequence has been submitted to GenBank as accession number U11034. Nucleotide 1 corresponds to the start codon of the 2406-bp ORF. The inverted triangles mark the positions of introns as deduced by comparison of the cDNA and genomic DNA sequences. The locations of single nucleotide changes in *ga1-6*, *ga1-7*, *ga1-8*, and *ga1-9* alleles are indicated by underlining, and the substituted bases are given above the sequences. The *ga1-4* allele contains a small deletion of 14 nucleotides from 2375 to 2388 as indicated by two arrows.

results of the complementation tests and the functional analyses, we demonstrate that this ORF encodes *ent*-kaurene synthetase A and that the protein is active when expressed both in Arabidopsis and in *Escherichia coli*. Immunoblot analysis and in vitro protein import experiments showed that the GA1 protein can be translocated into and processed in the chloroplasts.

RESULTS

Characterization of the *GA1* Gene

The genomic DNA clones (10 to 20 kb) and a partial cDNA clone (0.9 kb) corresponding to the Arabidopsis *GA1* locus were isolated previously (Sun et al., 1992). One additional *GA1* cDNA clone of 2.6 kb was obtained by screening 5 × 10^5 cDNA clones from a silique library of the Arabidopsis ecotype Columbia. DNA sequence analyses of the cDNA clones and the *GA1* genomic clones were performed to characterize the complete structure of the *GA1* locus (Figures 1 and 2). The 2.6-kb cDNA is nearly full length as was previously determined for the *GA1* mRNA, which is 2.8 kb (Sun et al., 1992). The ATG codon at position 48 to 50 of the 2.6-kb cDNA is most likely the translational start site for the GA1 protein because it is the first ATG codon and is followed by a long ORF of 2406 bp and a poly(A) tail (Figure 1). The predicted size of the protein from the amino acid sequence of this ORF is 93 kD, which is close to the initial size of the GA1 protein estimated by SDS-PAGE (86 kD), as discussed below. The 2.4-kb ORF spans ~7 kb of the genomic DNA that contains 15 exons and 14 introns (Figure 2). All introns contain 5′-GT and 3′-AG splice-junction consensus sequences. There is a putative TATA box (TATAAACA) located at nucleotides −287 to −280 upstream from the presumptive translational start codon and tandem repeats of polyadenylation signal (AATAAA) at nucleotides 117 to 128 downstream from the translational stop codon.

Koornneef et al. (1983) constructed a fine-structure genetic map of the *GA1* locus using nine *ga1* alleles, which were subsequently renamed in Sun et al. (1992). Three of these *ga1* alleles (*ga1-2*, *ga1-3*, and *ga1-4*) were generated by fast neutron bombardment, and six (*ga1-1*, *ga1-6*, *ga1-7*, *ga1-8*, *ga1-9*, and *ga1-10*) were generated by ethyl methanesulfonate mutagenesis (Koornneef et al., 1983). Table 1 and Figures 1 and 2 summarize the nature and position of eight of the nine *ga1* mutations. As expected, all ethyl methanesulfonate–induced mutations are single-base substitutions, whereas fast neutron bombardment generated a rearrangement (insertion or inversion) and deletions. Five of the mutations, *ga1-2* (inversion or

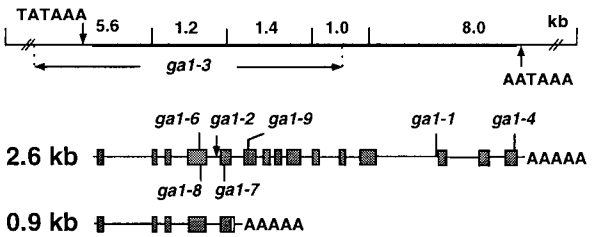

Figure 2. Physical Map of the *GA1* Locus.

The top horizontal line shows the HindIII restriction map, and the heavy line indicates the coding region of the *GA1* gene. The putative TATA box (TATAAA) and the poly(A) signal (AATAAA) are labeled by arrows. The location of the 5-kb deletion in the *ga1-3* mutant is indicated by two arrows. The diagrams below the restriction map depict the introns (lines) and exons (shaded boxes) derived from two cDNA clones (2.6 and 0.9 kb) of the *GA1* gene. The fifth exon of the 0.9-kb cDNA extends an additional 48 nucleotides (open box). The positions of mutations in *ga1-2* (inversion or insertion), *ga1-4* (14-bp deletion), *ga1-1*, *ga1-6*, *ga1-7*, *ga1-8*, and *ga1-9* (point mutations) are indicated along the diagram for the 2.6-kb cDNA.

insertion), *ga1-3* (5-kb deletion), *ga1-6*, *ga1-7*, and *ga1-8* (point mutations), were placed on the physical map as described previously (Sun et al., 1992). Mutant *ga1-7*, which defines one side of the genetic map, contained a point mutation in the most distal exon (exon 5) in the 0.9-kb cDNA (Figure 2). However, isolation of the 2.6-kb cDNA revealed that the 0.9-kb cDNA most likely resulted from either a cloning artifact or a premature termination of transcription in intron 6. Polymerase chain reaction (PCR) and DNA sequencing analyses were performed to determine the positions of three additional *ga1* alleles, *ga1-1*, *ga1-4*, and *ga1-9*. Two of these alleles, *ga1-1* and *ga1-4*, define the other side of the *GA1* genetic map, whereas *ga1-9* is located at the middle of the genetic map and overlaps with the *ga1-3* deletion mutation. Sequence analysis showed that *ga1-4* contains a small deletion of 14 nucleotides in the last exon (exon 15, Figures 1 and 2). *ga1-1* and *ga1-9* contain single base changes at the 3′ splice junction in intron 12 (AG to AA) and in exon 6 (TGG to TAG, amber codon), respectively. Mutations in all three alleles are located downstream from the coding sequences defined by the 0.9-kb cDNA (Figure 2). The results indicate that the 0.9-kb cDNA does not encode a functional GA1 protein.

The positions of various *ga1* mutations (Table 1 and Figure 2) correspond well to their locations on the genetic map (Koornneef et al., 1983), except that the *ga1-7* mutation is located between *ga1-2* and *ga1-9* on our physical map in contrast to being at one end of the genetic map. Using the recombination frequency between *ga1-6*, *ga1-7*, and *ga1-8*, we previously estimated the recombination frequency within the *GA1* locus to be $\sim 10^{-5}$ centimorgans (cM) per nucleotide (Sun et al., 1992). We reexamined this value using *ga1-6*, *ga1-9*, and *ga1-4*, and the recombination frequency within the *GA1* locus was $\sim 1.2 \times 10^{-5}$ cM per nucleotide. This is in good agreement with the average recombination frequency of the Arabidopsis genome ($\sim 5.2 \times 10^{-6}$ cM per nucleotide; Hauge et al., 1993).

Complementation Analysis

To test if the 2.4-kb ORF of the *GA1* cDNA encodes a functional protein in Arabidopsis, we expressed the *GA1* cDNA in *ga1-3* deletion mutant plants. The 2.6-kb *GA1* cDNA was fused transcriptionally to a cauliflower mosaic virus (CaMV) 35S promoter in both sense (pGA1-45) and antisense (pGA1-47) orientations in the binary vector pBIN19. To maximize the expression of the *GA1* cDNA, the 2.4-kb coding sequence was also fused translationally to a CaMV 35S promoter with duplicated enhancer and 5′ nontranslated regions (NTR) from tobacco etch virus (TEV). TEV–NTR has been shown to enhance efficiency of translation in vivo and in vitro (Carrington and Freed, 1990). The DNA cassette containing the CaMV 35S–TEV–NTR–GA1 gene fusion was inserted into the binary vector pBIN19, and the resulting plasmid was named pGA1-49. Gene fusions in plasmids pGA1-45, pGA1-47, and pGA1-49 were each transferred into the *ga1-3* genome via Agrobacterium-mediated transformation. Several (three, seven, and eight for pGA1-45, pGA1-47, and pGA1-49, respectively) independent kanamycin-resistant (Km^r) transgenic plants (T_1 generation) were regenerated. All T_1 plants derived from the sense GA1 constructs, pGA1-45 and pGA1-49, set seeds in the absence of exogenous GA. Seeds (ranging from 30 to 400) from each T_1 transgenic line showed 100% linkage of the

Table 1. The Nature and Position of Mutations in Various *ga1* Mutants

Mutant	Nature of Mutation[a]	Position in Coding Sequence	Position in Genomic Sequence
ga1-1	AG→AA 3′ Splice junction		Intron 12
ga1-2	≥3.4-kb Insertion or inversion		Intron 4
ga1-3	5-kb Deletion	~1 kb 5′ Upstream of ATG to 1621	~1 kb 5′ Upstream of ATG to exon 11
ga1-4	14-Nucleotide deletion	2375 to 2388	Exon 15
ga1-6	TCT→TTT Ser Phe	452	Exon 4
ga1-7	GAA→AAA Glu Lys	631	Exon 5
ga1-8	GGA→AGA Gly Arg	457	Exon 4
ga1-9	TGG→TAG Trp stop	818	Exon 6

[a] Underlining denotes base substitution in each mutant.

$GA1^+$ and Km^r phenotypes; most of these lines (all pGA1-45 lines and seven of the pGA1-49 lines) segregated ~3:1 in relation to the GA^-/kanamycin-sensitive (Km^s) phenotype (T_2 generation). All T_2 generation $GA1^+/Km^r$ transgenic plants grew as tall as wild-type plants and set seeds without exogenous GA treatment. This result indicated that the 2.4-kb ORF encodes an active GA1 protein, which complemented the *ga1-3* mutation in these transgenic plants. Seven Km^r T_1 plants derived from the control pGA1-47 (antisense GA1) were regenerated. Similar to the phenotype of the original *ga1-3* plants, these transgenic plants all required exogenous GA_3 treatment for vegetative growth, flowering, and seed set.

Functional Analysis of the GA1 Protein

The *GA1* gene product could either be a cyclase, *ent*-kaurene synthetase A or B, or a regulatory protein. To study the function of the GA1 protein, the full-length (2.6-kb) and truncated (0.9-kb) *GA1* cDNAs were overexpressed in *E. coli*. Figure 3 shows that the 2.6-kb cDNA in pGA1-43 encodes the full-length GA1 protein of 86 kD (lane 5), and the 0.9-kb cDNA in pGA1-40 produces a truncated GA1 protein of 30 kD (lane 2). Both the 30- and the 86-kD proteins were purified from *E. coli* extracts by isolation of inclusion bodies (Marston, 1987), followed by SDS-PAGE and electroelution. The gel-purified proteins were detected as single bands on an SDS–polyacrylamide gel by Coomassie blue staining (Figure 3, lanes 3 and 6). The 30-kD protein was further examined by N-terminal sequence analysis and was shown to have the six–amino acid sequence at the N terminus that was predicted by the cDNA sequence, whereas the N terminus of the 86-kD protein was blocked. Antibodies to the 30- and the 86-kD GA1 proteins were obtained by immunization of rabbits with the gel-purified proteins.

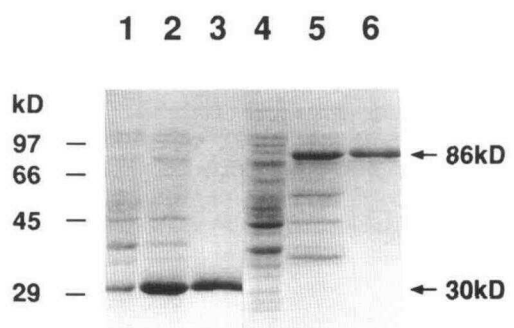

Figure 3. Overexpression of the Arabidopsis *GA1* Gene in *E. coli* Using the T7 RNA Polymerase Expression System.

Lanes 1 and 4 contain uninduced (without isopropyl β-D-thiogalactopyranoside) crude extracts, and lanes 2 and 5 contain induced (with isopropyl β-D-thiogalactopyranoside) inclusion body fractions from cells carrying the 0.9- and the 2.6-kb *GA1* cDNAs, respectively. Lanes 3 and 6 contain gel-purified GA1 proteins of 30 (truncated) and 86 kD, respectively. Numbers at left represent the positions and sizes of molecular standards.

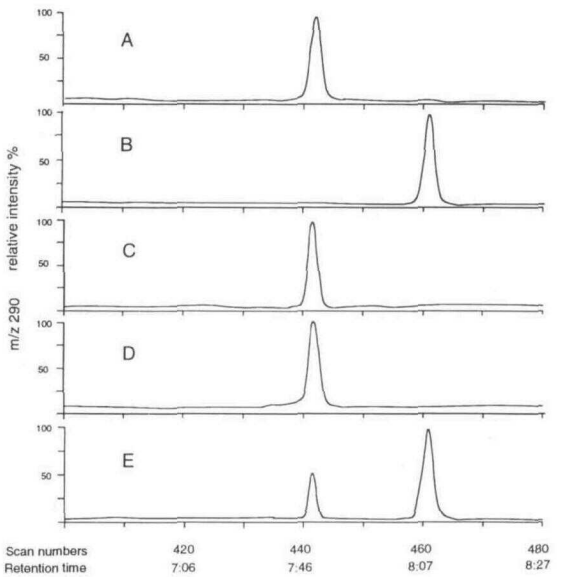

Figure 4. GC–MS Identification of GGol and Copalol from Hydrolyzed Methanol Extracts of *E. coli*.

Mass chromatograms at a mass-to-charge ratio (*m/z*) of 290 (molecular ion of GGol and copalol) are shown in **(A)** to **(E)**. Retention time is in minutes.
(A) and **(B)** Authentic GGol and copalol standards, respectively.
(C) to **(E)** Hydrolyzed *E. coli* extracts from cells carrying only pACCRT-E (GGPP synthase), pACCRT-E and pGA1-40 (30-kD truncated GA1 protein), and pACCRT-E and pGA1-43 (86-kD GA1 protein), respectively.

Sandmann and Misawa (1992) demonstrated that the *CrtE* gene of *Erwinia uredovora* encodes GGPP synthase, which catalyzes the conversion of farnesyl pyrophosphate to GGPP. *E. coli* cells harboring the *CrtE* gene accumulate large amounts of GGPP (Sandmann and Misawa, 1992). This is in contrast to normal *E. coli* cells that only produce trace amounts of GGPP. The plasmid pACCRT-E, which contains the *CrtE* gene, was cotransformed with either the control plasmid pGA1-40 (0.9-kb *GA1* cDNA) or pGA1-43 (2.6-kb *GA1* cDNA) into *E. coli* cells. GGPP and CPP were extracted from cells carrying pACCRT-E alone, pACCRT-E and pGA1-40, or pACCRT-E and pGA1-43, and the hydrolyzed extracts were analyzed using gas chromatography–mass spectrometry (GC–MS). The products were identified by full-scan GC–MS. Figures 4A and 4B show the pattern of the mass chromatography with a mass-to-charge ratio of 290, which is the ratio of the molecular ions of geranylgeraniol (GGol) and copalol. The extracts from cells harboring only pACCRT-E or both pACCRT-E and pGA1-40 contained high levels of GGol but did not have any detectable copalol (Figures 4C and 4D). In contrast, copalol accumulated to a quite high level in cells carrying both pACCRT-E and pGA1-43 and consequently producing both GGPP synthase and the 86-kD GA1 protein (Figure 4E). These results indicate that the 86-kD protein encoded by the 2.4-kb ORF of the *GA1* cDNA is the enzyme *ent*-kaurene synthetase A, which catalyzes the conversion of GGPP to CPP. The truncated 30-kD GA1 protein does not have this enzyme activity.

GA1 Protein Level in Wild-Type and Transgenic Lines Containing Various Gene Fusions

The levels of GA1 proteins in both transgenic Arabidopsis plants containing sense or antisense GA1 constructs were compared to the level in wild-type plants (ecotype Landsberg *erecta*) by immunoblot analysis (Figure 5). Supernatant fractions, which contained most of the *ent*-kaurene synthetase activity, were obtained by tissue extraction and centrifugation (Bensen and Zeevaart, 1990). A major protein band of 76 kD was labeled by the GA1 antibodies in three overexpression lines tested (Figure 5, lanes 3, 4, and 5). This protein accumulated at higher levels in the plants containing the CaMV 35S–TEV–NTR–GA1 construct (lanes 4 and 5) than in the plants carrying CaMV 35S–GA1 (lane 3). This protein is absent in lanes 2 and 6, which contain proteins extracted from a transgenic line carrying the antisense construct and from wild-type plants, respectively. The sensitivity of this analysis could detect as little as ~1 ng of the gel-purified 86-kD GA1 protein produced in *E. coli*. Because the endogenous *GA1* gene is expressed at extremely low levels (Sun et al., 1992), it is not surprising that the GA1 antibodies could not detect the endogenous GA1 protein in wild-type plants.

Protein Import into Isolated Pea Chloroplasts

The first 50 N-terminal amino acids of the GA1 protein are rich in serine (26%) and threonine (12%) with an estimated pI of 10.2. These properties are common features of the transit peptides of many chloroplast proteins (Keegstra et al., 1989). A ^{35}S-methionine/cysteine–labeled GA1 protein of 86 kD was synthesized in vitro using SP6 RNA polymerase and a rabbit reticulocyte translation system (Figure 6, lane 3). The size of this in vitro–translated protein is the same as that expressed in *E. coli* cells (Figure 6, lane 1). When the 86-kD in vitro–translated product was incubated with isolated pea chloroplasts, it was processed to a smaller, 76-kD protein that was protected from digestion by externally added protease (Figure 6, lane 4). This protein was degraded by the protease when chloroplasts were disrupted by 0.1% Triton X-100 (Figure 6, lane 5). Immunoblot analysis showed that the GA1 protein produced by the *GA1* cDNA in transgenic plants migrated as a 76-kD protein (Figure 6, lane 2). These results suggest that the GA1 proteins are targeted to and processed in chloroplasts in the plant.

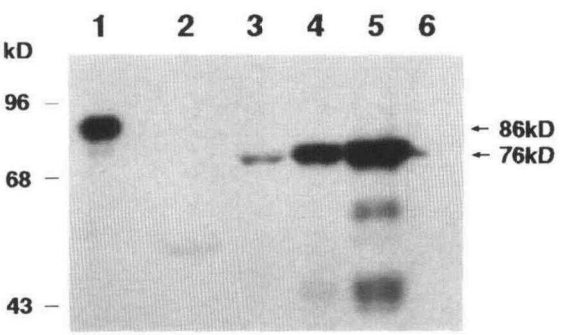

Figure 5. Immunoblot Analysis of GA1 Protein Levels in Soluble Protein Fractions from Arabidopsis Using GA1 Antisera.

Protein extracts from 2-week-old Arabidopsis seedlings were fractionated by centrifugation at 100,000*g*, and the supernatant fraction was separated by electrophoresis on an 8% SDS–polyacrylamide gel. The protein gel blot was incubated with 30-kD GA1 antisera and peroxidase-conjugated goat anti–rabbit antisera, and the protein was detected using an enhanced chemiluminescence reagent followed by autoradiography. The blot contains 15 ng of gel-purified 86-kD protein produced from *E. coli* carrying pGA1-43 (lane 1); 50 μg of the 100,000*g* supernatant fractions from transgenic plants containing CaMV 35S promoter–antisense GA1 (lane 2); CaMV 35S promoter–GA1 (lane 3); CaMV 35S–TEV–NTR–GA1 (lanes 4 and 5); Landsberg *erecta* (lane 6). Numbers at left indicate the positions and sizes of molecular standards. Numbers at right indicate sizes of GA1 protein produced from *E. coli* (86 kD) and transgenic Arabidopsis plants (76 kD).

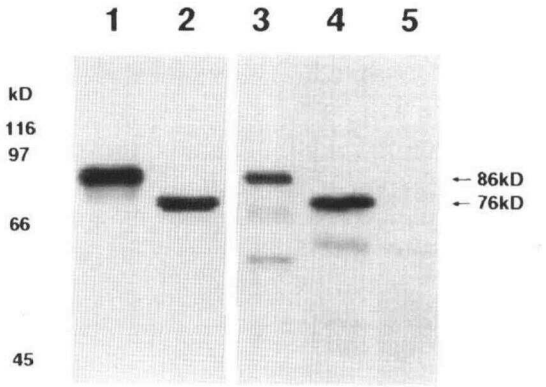

Figure 6. Import of GA1 Protein into Isolated Pea Chloroplasts.

Lanes 1 and 2 are an immunoblot of 10 ng of gel-purified 86-kD protein produced by *E. coli* carrying pGA1-43 and 15 μg of the 100,000*g* supernatant fraction from an Arabidopsis transgenic line carrying the CaMV 35S–TEV–NTR–GA1 gene fusion, respectively. Lanes 3 to 5 are from a fluorogram of the ^{35}S-labeled GA1 protein subjected to different treatments. Lane 3 is an aliquot of the total in vitro–translated products containing the ^{35}S-labeled GA1 protein. Lanes 4 and 5 are the labeled protein sample after uptake by isolated intact pea chloroplasts, followed by protease treatment in the absence or presence of 0.1% Triton X-100, respectively. Numbers at left represent the positions and sizes of molecular standards. Numbers at right represent sizes of unprocessed (86 kD) and processed (76 kD) GA1 protein.

Sequence Comparison with Other Terpene Cyclases

Comparison of the predicted GA1 amino acid sequence (802 amino acids) with sequences in the GenBank data base revealed that stretches of 124 amino acids (328 to 451) and 165 amino acids (334 to 498) in the GA1 protein share sequence similarities of 72% (36% identity) and 72% (32% identity) with that of tobacco sesquiterpene cyclase (Facchini and Chappell, 1992) and a monoterpene cyclase, spearmint limonene

synthase (Colby et al., 1993), respectively. A diterpene cyclase, castor bean casbene synthase (Colby et al., 1993), also has a sequence similarity of 72% (30% identity) with a stretch of 182 amino acids (329 to 510) in the GA1 protein. Figure 7 shows alignment of the predicted GA1 sequence (327 to 604) to partial peptide sequences of these three terpene cyclases. The sequence DDXXD, which was proposed to function in binding the divalent metal ion–pyrophosphate complex of the prenyl substrate (Ashby et al., 1990), is absent in the GA1 protein sequence. This sequence is highly conserved among the other three terpene cyclases and several other prenyltransferases (Jennings et al., 1991; Facchini and Chappell, 1992). These enzymes all catalyze the condensation reaction of allylic pyrophosphates to produce cyclized terpenes or higher prenyl pyrophosphates. In contrast, GA1 catalyzes the cyclization reaction without removal of the pyrophosphate group.

An alternative aspartate-rich motif, DXDDTA, was identified at residues 377 to 382 in the GA1 sequence. This sequence is also found in squalene–hopene cyclases isolated from *Zymomonas mobilis* (GenBank, EMBL, and DDBJ accession number X73561) and *Bacillus acidocaldarius* (Ochs et al., 1992). These enzymes catalyze the direct cyclization of a triterpene, squalene, to form hopanoids; the substrate squalene does not contain pyrophosphate. The common catalytic property between these enzymes and the GA1 protein is the ring closure reaction of terpenoid compounds. Although the squalene–hopene cyclases do not have large regions of sequence similarity with the GA1 protein, the DXDDTA motif may be involved in the catalytic activity of these enzymes.

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tobsqpc    8  KLADTLNLID IIERLGISYH FEKEIDEILD QI..YNQN..  .SN.....CN
casbene  112  DSVETVILID LLCRLGVSYH FENDIEELLS KI..FNSQ..  .PDLVDEKEC
limonene 105  DQIRQLELID DLQRMGLSDH FQNEFKEILS SI..YLDHHY  YKNPFPKEER
    ga1  327  DLFEHIWIVD RLQRLGISRY FEEEIKECLD YVHRYWTDNG  ICWARCSHVQ
consensus      dl.e.....D .lqRlGiS..  Fe.eikE.Ld ....y.....  ..........

tobsqpc   48  DLCTSALQFR LLRQHGFNIS PEIFSKFQDE NG..KFKESL  ASDVLGLLNL
casbene  157  DLYTAAIVFR VFRQHGFKMS SDVFSKFKDS DG..KFKESL  RGDAKGMLSL
limonene 153  DLYSTSLAFR LLREHGFQVA QEVFDSFKNE EG..EFKESL  SDDTRGLLQL
    ga1  377  DIDDTAMAFR LLRQHGYQVS ADVFKNFEKE GEFFCFVGQS  NQAVTGMFNL
consensus      D...ta.aFR llRqHG.qvs .dvF..F..e .....F....  ...v.Gm.nL

tobsqpc   96  YEASHVRTHA DDILEDALAF STIHL.....  .ESAAPH..L  KSPLREQVTH
casbene  205  FEASHLSVHG EDILEEAFAF TKDYL.....  .QSSAVE..L  FPNLKRHITN
limonene 201  YEASPLLTEG ETTLESAREF ATKFL.....  .EEKVNEGGV  DGDLLTRIAY
    ga1  427  YRASQLAFPR EEILKNAKEF SYNYLLEKRE  REELIDKWII  MKDLPGEIGF
consensus      y.AS.l.... e.iL..A.eF s..yL.....  .ee.......  ..dL...i..

tobsqpc  138  ALEQCLHKGV PRVETRFFIS SIYDKEQSK.  ..........  .NNVLLRFAK
casbene  247  ALEQPFHSGV PRLEARKFID LYEADIECR.  ..........  .NETLLEFAK
limonene 245  SLDIPLHWRI KRPNAPVWIE WYRKRPD.M.  ..........  .NPVVLELAI
    ga1  477  ALEIPWYASL PRVETRFYID QYGGENDVWI  GKTLYRMPYV  NNNGYLELAK
consensus      aLeip.....  pRvetrf.Id .y....d...  ..........  .Nn..LelAk

tobsqpc  176  LDFNLLQMLH KQELAQVSRW WKDLDFVTTL  PYARDRVVEC  YFWALGVYFE
casbene  285  LDYNRVQLLH QQELCQFSKW WKDLNLASDI  PYARDRMAEI  FFWAVAMYFE
limonene 282  LDLNIVQAQF QEELKESFRW WRNTGFVEKL  PFARDRLVEC  YFWNTGIIEP
    ga1  527  QDYNNCQAQH QLEWDIFQKW YEENRL.SEW  GVRRSELLEC  YYLAAATIFE
consensus      .DyN..Qaqh q.E...f.kW .....l.s..  ...R..l.Ec  y..a.a.ife

tobsqpc  226  PQYSQARVML VKTISMISIV DDTFDAYGT
casbene  335  PDYAHTRMII AKVVLLISLI DDTIDAYAT
limonene 332  RQHASARIMM GKVNALITVI DDIYDVYGT
    ga1  576  SERSHERMVW AKSSVLVKAI SSSFGESSD
consensus      ...sh.Rm.. aK...l...i ...f.....
```

Figure 7. Sequence Alignment of the GA1 Protein Compared to Tobacco Sesquiterpene Cyclase, Casbene Synthase, and Limonene Synthase.

Uppercase letters in the consensus sequence indicate that all four proteins contain the same amino acid residue. When at least one letter in the first three peptides is the same as that of the GA1 protein, the consensus character is lowercase. Dots indicate that there is no homology between the first three proteins and the GA1 protein. The putative divalent metal ion–pyrophosphate complex binding site (DDXXD) is indicated by the box. The DXDDTA motif in the GA1 sequence is highlighted in boldface. tobsqpc, tobacco sesquiterpene cyclase.

DISCUSSION

In this study, we showed that the 2.4-kb ORF in the *GA1* cDNA produced an active protein that can complement the dwarf phenotype of the *ga1-3* mutant when expressed in the plant under the control of the CaMV 35S promoter. Functional analysis using an *E. coli* expression system indicated that the GA1 protein has *ent*-kaurene synthetase A activity, which catalyzes the conversion of GGPP to CPP. Because *ent*-kaurene synthetase A catalyzes the first committed step in GA biosynthesis, the deletion mutant *ga1-3* should not produce *ent*-kaurene or any GAs. However, M. Talon and J.A.D. Zeevaart (personal communication) have detected a low level of GAs in extracts isolated from the *ga1-3* mutant plant. This discrepancy could be explained if there is either a *GA1* homolog in Arabidopsis or an alternative pathway in GA biosynthesis that would possibly bypass the step catalyzed by the GA1 protein. DNA gel blot analysis using a partial *GA1* cDNA as a probe under low-stringency hybridization conditions showed an additional DNA fragment that is present in both wild-type Arabidopsis ecotype Landsberg *erecta* and in the *ga1-3* deletion mutant (data not shown). This gene could encode either a *GA1* homolog or another related terpene cyclase.

Three independent transgenic *ga1-3* lines containing the CaMV 35S–GA1 gene fusion developed normally as wild-type Arabidopsis plants. One of these lines tested accumulated a higher level of GA1 protein than did the wild-type plants (Figure 5, lane 3). Several independent transgenic *ga1-3* lines containing the CaMV 35S–TEV–NTR–GA1 gene fusion showed slightly aberrant leaf expansion and/or abnormal flower development and a partially sterile phenotype (data not shown). Two of these lines produced extremely high levels of the GA1 proteins (Figure 5, lanes 4 and 5). Future studies will use genetic analysis to determine if the aberrant phenotype is linked to overexpression of the *GA1* gene. The levels of GA intermediates in these transgenic plants will also need to be determined by GC–MS.

There are several possible reasons why overexpression of the GA1 protein would not drastically affect GA biosynthesis and plant growth. Duncan and West (1981) showed that CPP derived from ^{14}C-GGPP is more rapidly converted to *ent*-kaurene than exogenously added ^{3}H-CPP in protein fractions containing both *ent*-kaurene synthetases A and B enzyme activities. They suggested that the metabolism of GGPP into *ent*-kaurene occurs by separate but interacting proteins. CPP derived from the A enzyme was thought to be channelled to the B enzyme catalytic site for conversion to *ent*-kaurene. Low

levels of the B enzyme may be rate limiting in plants that overexpress the GA1 protein. As a result of the size and complexity of the GA biosynthetic pathway, it is probable that other steps further downstream are also regulated. This would also prevent extremely aberrant phenotypes. Improper sites for and timing of expression of the *GA1* gene by the viral promoter in the transgenic plants may also be a factor. Maximum *ent*-kaurene synthesizing activity has been found in rapidly developing tissues, such as the shoot apex and immature seeds (Chung and Coolbaugh, 1986). The transgenes are under the control of the CaMV 35S promoter, which is more active in seedlings and flowers than in other mature vegetative tissues (Caspar and Quail, 1993). We are currently in the process of examining the pattern of expression of the endogenous *GA1* gene using a *GA1* promoter-β-glucuronidase gene fusion. The data resulting from this analysis will be used to design organ-specific promoters and *GA1* cDNA gene fusions to manipulate the GA biosynthesis in specific organs.

Our data from the chloroplast import experiment indicate that GA1 protein can be translocated into plastids, and it is likely to be a stromal protein because it is present in the 100,000*g* supernatant fraction. This is consistent with the results of H. Aach and J.L. Graebe (personal communication) that *ent*-kaurene synthetases A and B are present in the chloroplast fraction of wheat seedlings. In the second stage of GA biosynthesis, oxidation and ring contraction occur in five steps to convert *ent*-kaurene to GA_{12}-aldehyde. Enzymes involved in the second stage of the pathway have been localized in the endoplasmic reticulum membrane fraction of pea and pumpkin seeds (Graebe, 1982; Hafemann, 1985). The mechanism responsible for transferring *ent*-kaurene from the chloroplasts to the endoplasmic reticulum is not known. It could be via direct contact between the organellar membranes or through vesicle-mediated transport. *ent*-Kaurene synthetase B activity has been reported to be in the stroma of chloroplasts of several plant species (West et al., 1982), but it required the presence of chloroplast membranes for maximum activity (Railton et al., 1984). Railton et al. (1984) suggested that the B enzyme is weakly associated with chloroplast membranes, and this association might facilitate the production of hydrophobic *ent*-kaurene for further oxidation by membrane-bound enzymes. The subcellular localization of the GA pathway after the formation of GA_{12}-aldehyde is not clear. GA biosynthesis may be partially regulated by separating various steps in the pathway into different organelles.

Grafting experiments using various pea mutants defective in GA biosynthesis suggest that the bioactive GA for internode elongation, GA_1, is not transported, but its precursor, GA_{20}, is the major transported GA in peas (Reid et al., 1983; Proebsting et al., 1992). The regulatory mechanisms and the site of GA biosynthesis in response to endogenous developmental programs and environmental stimuli remain to be elucidated. By using the cloned *GA1* cDNA, we are in the process of examining the regulation of *ent*-kaurene biosynthesis. Our study will provide information on the site and regulation of the first committed step in the GA biosynthetic pathway.

METHODS

Plant Materials

Arabidopsis thaliana ga1 mutants were obtained from M. Koornneef (Agricultural University, Wageningen, The Netherlands).

Isolation of the 2.6-kb *GA1* cDNA Clone

The 2.6-kb cDNA clone pGA1-29 was isolated by screening a cDNA library that was constructed from RNA isolated from green siliques of Arabidopsis ecotype Columbia (Giraudat et al., 1992) using the ^{32}P-labeled 0.9-kb *GA1* cDNA (pGA1-24; Sun et al., 1992) as the hybridization probe.

Plasmid Construction

The DNA sequence around the first ATG codon of the *GA1* gene was modified to contain either an AflIII site or an NcoI site by polymerase chain reaction (PCR). Conversion to the AflIII site did not change the coding sequence. The introduction of the NcoI site at the ATG codon created a single base change in the second codon (TCT to GCT; Ser to Ala). The PCR-amplified 0.5-kb AflIII-SphI and NcoI-SphI DNA products were cloned into the AflIII-SphI sites of pUC19 (pGA1-41) and NcoI (converted from HindIII site)-SphI sites of pUC18 (pGA1-32). The cloned PCR products were sequenced to ensure that no mutations were introduced during amplification. The rest of the coding sequence for the full-length GA1 protein was excised from pGA1-29 by SphI and EcoRI (blunt ended by the Klenow fragment of DNA polymerase I) and ligated to SphI and HincII sites of pGA1-41 to create the full-length *GA1* cDNA with an AflIII site at the initiation codon. The entire coding region of the *GA1* cDNA was excised by AflIII and BamHI as a 2.5-kb DNA fragment and inserted into NcoI and BamHI sites of the pET-8c vector (Studier et al., 1990). The resulting plasmid, which contained 2.5-kb *GA1* cDNA under control of the T7 promoter by translational fusion, was named pGA1-43. This plasmid was used to express the full-length GA1 protein in *Escherichia coli* cells. The rest of the coding sequence in the 0.9-kb cDNA was excised from pGA1-24 by SphI and BamHI and ligated to SphI and BamHI sites of pGA1-32. The 0.9-kb coding sequence was excised by NcoI and BamHI and was cloned into pET-8c vector. This plasmid was named pGA1-40, which was used to express a 30-kD truncated GA1 protein in *E. coli* cells.

The 2.5-kb AflIII-BamHI *GA1* cDNA was fused to cauliflower mosaic virus (CaMV) 35S promoter with dual enhancer and 5′ nontranslated region (NTR) from tobacco etch virus (TEV) by the following procedure. A 1.2-kb HindIII cassette containing the CaMV 35S promoter with the dual enhancer TEV-NTR and CaMV 35S poly(A) signal was excised from pRTL2 (Restrepo et al., 1990) and ligated to the HindIII site of pBluescript SK+ vector (Stratagene). The 2.5-kb AflIII-BamHI cDNA was inserted into NcoI-BamHI sites of the above-mentioned plasmid so that the *GA1* cDNA was in a sense orientation behind the CaMV 35S promoter and the TEV-NTR leader sequence (pGA1-48). A 2.6-kb EcoRI-BamHI fragment carrying TEV-NTR-GA1 DNA was excised from pGA1-48 and incubated with T4 DNA polymerase to create blunt ends. This DNA was then ligated into the HincII site of pSP64 (poly[A]; Promega) to generate *GA1* transcripts with poly(A) tails in vitro using SP6 RNA polymerase (pGA1-84). A 4-kb SmaI-SalI fragment of pGA1-48 containing the CaMV 35S-TEV-NTR-GA1 gene fusion was inserted

into the SmaI-SalI sites of the binary vector pBIN19 (Bevan, 1984), and the resulting plasmid was named pGA1-49.

The 2.6-kb cDNA in pGA1-29 is located in the EcoRI site of pBluescript SK+ and is in an antisense orientation behind the T7 promoter. This plasmid was cut with EcoRI, religated, and screened for plasmids with inserts in opposite orientation. The resulting plasmid was named pGA1-30. A 2.6-kb *GA1* cDNA was excised from pGA1-29 and pGA1-30 by XbaI and KpnI restriction enzymes and inserted into XbaI and KpnI sites located between the CaMV 35S promoter and the nopaline synthase (*nos*) terminator in pBIN19–35S. The vector pBIN19–35S was a gift from M. Conkling (North Carolina State University, Raleigh, NC) and was created by inserting a *nos* terminator into pWPF126 (Fitzmaurice et al., 1992). The resulting plasmids were named pGA1-45 (sense orientation) and pGA1-47 (antisense).

DNA Sequencing Analysis of Wild-Type and Mutant *GA1* DNA

DNA sequences of *GA1* genomic DNA and cDNA were obtained using the dideoxy method (Ausubel et al., 1990) with Sequenase version 2.0 T7 DNA polymerase (U.S. Biochemical Corp.) and both single- and double-stranded DNA templates. The 1.4-kb HindIII DNA in the *ga1-9* mutant was amplified by PCR and reamplified by asymmetric PCR, and the single-stranded DNA templates were sequenced directly (Innis et al., 1990). The 1.4-kb DNA fragments spanning intron 12 to exon 15 were amplified from genomic DNA isolated from *ga1-1* and *ga1-4* by PCR. These PCR-amplified DNA products were cloned into the SmaI site of the pBluescript SK+ vector, and DNA sequences were obtained by using double-stranded DNA templates isolated from several independent clones.

Agrobacterium tumefaciens–Mediated Transformation of Arabidopsis Root Explants

The transformation procedure was as described previously (Valvekens et al., 1988) with slight modifications (Sun et al., 1992). pGA1-45, pGA1-47, and pGA1-49 were introduced into Agrobacterium LBA4404 by electroporation (Ausubel et al., 1990). Stability of the plasmid insert in LBA4404 was tested by restriction digestion and gel electrophoresis of plasmid DNA purified by the NaOH-SDS minipreparation procedure (Ausubel et al., 1990).

A fresh overnight culture of LBA4404 carrying individual plasmids was used to infect root explants of 4-week-old *ga1-3* mutants. Kanamycin-resistant (Km^r) transgenic plants were regenerated as described previously (Valvekens et al., 1988). Seeds of transgenic plants were germinated on Murashige and Skoog agar (Life Technologies, Inc., Grand Island, NY) plates containing kanamycin (50 μg/mL). Nongerminating seeds after 8 days were transferred onto Murashige and Skoog agar plates containing 100 μM GA_3 and 50 μg/mL kanamycin to score for GA^+/Km^r and GA^-/kanamycin sensitive (Km^s) segregation.

Overexpression of GA1 Proteins in *E. coli* and the Procedure for Generating GA1 Antibodies

The pGA1-40 and pGA1-43 constructs were transformed into DE3 lysogenic *E. coli* BL21(DE3) (Studier et al., 1990). The expression of the *GA1* cDNA was induced by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside at $A_{600\ nm}$ of 0.8 with 2-hr incubation at 37°C. Cell cultures (30 mL) were harvested by centrifugation, washed, and resuspended in 10 mL of 50 mM Tris, pH 8.0, and 2 mM EDTA. The cells were sonicated on ice with a microtip probe (Heat Systems-Ultrasonics, Inc., Plainview, NY) at a setting of 4, with four 20-sec pulses. The sonicate was mixed with 1% Triton X-100, incubated on ice for 5 min, and then centrifuged at 12,000*g* for 10 min at 4°C to isolate inclusion bodies (Marston, 1987, with slight modification).

The 30- and the 86-kD GA1 proteins were purified from the inclusion body fraction of *E. coli* extracts by SDS-PAGE and electroelution with the Electro-Separation system (Schleicher & Schuell). The purified proteins were detected as single bands on SDS–polyacrylamide gels by Coomassie Brilliant Blue R 250 staining. Rabbit antibodies to either the 30- or 86-kD GA1 protein were obtained by subcutaneous injection of gel-purified proteins in complete Freund's adjuvant (Harlow and Lane, 1988). For N-terminal sequence analysis, proteins were fractionated by SDS-PAGE and then transferred to an Immobilon membrane (Millipore Corp., Bedford, MA) in Tris-glycine and 10% methanol. The membrane was first stained with Ponceau S and destained in deionized water; the 30- and 86-kD protein bands were excised for N-terminal sequence analysis (conducted by D. Klapper at University of North Carolina, Chapel Hill, NC).

Coexpression of the *GA1* cDNA and Geranylgeranyl Pyrophosphate Synthase Gene in *E. coli* Cells

For detection of the accumulation of copalyl pyrophosphate (CPP), pGA1-43 and pACCRT-E, containing the 2.5-kb *GA1* cDNA and the geranylgeranyl pyrophosphate (GGPP) synthase gene, *CrtE*, respectively, were cotransformed into *E. coli* HMS174(DE3). As a control, pGA1-40, containing the truncated *GA1* cDNA, and pACCRT-E plasmids were also cotransformed into HMS174(DE3). For each 200 mL of culture, 4 mL of fresh overnight culture was inoculated into 200 mL of Luria-Bertani broth with 30 μg/mL chloramphenicol and 100 μg/mL ampicillin and incubated at 37°C with vigorous shaking. When the $A_{600\ nm}$ reached 0.9, 200 μL of 0.1 M isopropyl β-D-thiogalactopyranoside was added, and the culture was incubated at 37°C for an additional hour. The cells were pelleted by centrifugation, washed with 100 mL of 10 mM Tris, pH 8.0, 0.1 M NaCl, and 1 mM EDTA, pH 8.0, and freeze dried.

Extraction of GGPP and CPP from *E. coli* Cells

Freeze-dried cells were resuspended in 1 mL of H_2O and extracted three times with 2 mL of methanol at 0°C. After centrifugation, the clear methanol–water extracts were concentrated in vacuo by rotary evaporation at 30°C. A small amount of water was added to the concentrated solution and concentrated again to remove any remaining methanol from the aqueous solution. The final residue was diluted in 2 mL of 25 mM Na_2CO_3 and extracted three times with 1 mL of hexane. The resulting aqueous phase was collected for gas chromatography–mass spectrometry (GC–MS) analysis.

Hydrolysis of Pyrophosphate Solution and Analysis of Geranylgeraniol and Copalol by GC–MS Analysis

One-fifth of the pyrophosphate solution was hydrolyzed with 18 units of bacterial alkaline phosphatase (Takara Shuzo Co. Ltd., Kyoto, Japan) in 100 mM Tris-HCl, pH 9.0, and 2 mM $MgCl_2$ at 30°C for 15 hr, followed by three hexane extractions. The pooled hexane extracts were concentrated by gentle N_2 flow and dissolved in 200 μL of hexane,

and 1 μL of this sample was analyzed by full-scan GC–MS. GC–MS was performed with a Finnigan MAT INCOS 50 mass spectrometer coupled to an HP-5890A gas chromatograph (Finnigan MAT, San Jose, CA) equipped with a capillary column (DB-1, 0.32 mm i.d. × 15 m; J & W Scientific Inc., Folsom, CA) as described previously (Saito et al., 1991). Experiments were repeated twice. Authentic geranylgeraniol (GGol) and copalol were gifts from T. Takigawa (Kurare Central Research Institute, Kurashiki, Japan) and T. Nakano (Venezuela Science Institute, Caracas, Venezuela), respectively.

Immunoblot Analyses

Proteins from 2-week-old Arabidopsis seedlings were extracted and fractionated by centrifugation at 10,000*g* for 10 min and then at 100,000*g* for 90 min at 4°C (Bensen and Zeevaart, 1990). The 100,000*g* supernatant fractions (50 μg each) were loaded on an 8% SDS–polyacrylamide gel, electrophoresed, and transferred to a GeneScreen membrane (Du Pont–New England Nuclear). Immunoblot analysis was performed as described previously (Sambrook et al., 1989). The membrane was incubated with 1000-fold diluted 30-kD GA1 antisera (primary antibody) and then with 2500-fold diluted peroxidase-conjugated goat anti–rabbit antisera (secondary antibody; Sigma); the protein was detected using an enhanced chemiluminescence reagent (Amersham) and was followed by autoradiography.

Import of in Vitro–Synthesized GA1 Protein into Intact Pea Chloroplasts

Plasmid pGA1-84, which contains TEV–NTR–GA1 cDNA, was linearized by incubation with EcoRI and was used as a template for in vitro transcription in the presence of diguanosine triphosphate (Pharmacia) and SP6 RNA polymerase (New England BioLabs, Beverly, MA; Krainer et al., 1984). The resulting 5′-capped *GA1* transcripts were translated in vitro using a Promega rabbit reticulocyte translation system with ^{35}S-labeled methionine/cysteine (ICN Biomedicals, Irvine, CA) according to the Promega manual. The translation mixture was centrifuged at 100,000*g* for 15 min at 4°C. The postribosomal supernatant was used for import experiments. Protein import into intact pea chloroplasts was performed as described previously (Grossman et al., 1982) with slight modification (Kohorn et al., 1986). After incubation with isolated pea chloroplasts, 200 μg/mL protease type X (thermolysin; Sigma) was added to degrade proteins not sequestered by the intact chloroplasts. Triton X-100 (0.1%) was added to one-tenth of the sample during thermolysin treatment. Intact chloroplasts were repurified by centrifugation through 35% Percoll before being analyzed on SDS–polyacrylamide gels and autoradiographed.

Peptide Sequence Comparison

The BLAST network service (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, MD) and the FASTA and BESTFIT Genetics Computer Group programs (University of Wisconsin, Madison, WI) were used to search for sequence homology between the peptides. The sequence alignment was generated by using the PILEUP and LINEUP in the Genetics Computer Group program.

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