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The *GA2* Locus of *Arabidopsis thaliana* Encodes *ent*-Kaurene Synthase of Gibberellin Biosynthesis

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The *ga2* mutant of *Arabidopsis thaliana* is a gibberellin-deficient dwarf. Previous biochemical studies have suggested that the *ga2* mutant is impaired in the conversion of copalyl diphosphate to *ent*-kaurene, which is catalyzed by *ent*-kaurene synthase (KS). Overexpression of the previously isolated KS cDNA from pumpkin (*Cucurbita maxima*) (*CmKS*) in the *ga2* mutant was able to complement the mutant phenotype. A genomic clone coding for KS, *AtKS*, was isolated from *A. thaliana* using *CmKS* cDNA as a heterologous probe. The corresponding *A. thaliana* cDNA was isolated and expressed in *Escherichia coli* as a fusion protein. The fusion protein showed enzymatic activity that converted [^{3}H]copalyl diphosphate to [^{3}H]*ent*-kaurene. The recombinant AtKS protein derived from the *ga2–1* mutant is truncated by 14 kD at the C-terminal end and does not contain significant KS activity in vitro. Sequence analysis revealed that a C-2099 to T base substitution, which converts Gln-678 codon to a stop codon, is present in the *AtKS* cDNA from the *ga2–1* mutant. Taken together, our results show that the *GA2* locus encodes KS.

GAs are a group of diterpene compounds, some of which are plant-growth regulators that control many aspects of plant development, such as seed germination, shoot elongation, and flower development. A number of GA-responsive dwarf mutants that are deficient in the biosynthesis of active GAs have been characterized in various plant species (for a recent review, see Hedden and Kamiya, 1997; Ross et al., 1997). Biochemical characterization of these mutants has contributed to the elucidation of GA biosynthetic pathways. *ent*-Kaurene is an early intermediate in GA biosynthesis and is synthesized by the cyclization of GGDP via CDP. In plants, this two-step cyclization involves two distinct enzymes: CPS catalyzes the cyclization of GGDP to CDP, which is then converted to *ent*-kaurene by KS. CPS and KS were formally called *ent*-kaurene synthases A and B, respectively (Hedden and Kamiya, 1997). Biochemical studies suggested that CPS and KS may interact with each other (Duncan and West, 1981) and that these enzymes are localized in plastids (Railton et al., 1984; Sun and Kamiya, 1994; Aach et al., 1995). There is some evidence that *ent*-kaurene biosynthesis is controlled by environmental conditions such as photoperiod (Zeevaart and Gage, 1993) and temperature (Moore and Moore, 1991), as well as during plant development (Chung and Coolbaugh, 1986; Silverstone et al., 1997).

From *Arabidopsis thaliana* six GA-responsive dwarf mutants (*ga1* through *ga6*) have been isolated and characterized (Koornneef and van der Veen, 1980; Sponsel et al., 1997). Each mutant is blocked in a specific step in GA biosynthesis. The *GA1* locus has been cloned (Sun et al., 1992) and shown to encode CPS (Sun and Kamiya, 1994). The *GA4* and *GA5* genes code for dioxygenases involved in later steps of the GA biosynthetic pathway (Chiang et al., 1995; Xu et al., 1995). The *GA2*, *GA3*, and *GA6* loci may encode enzymes catalyzing steps in the GA biosynthetic pathway or regulators of these enzymes.

The *ga2–1* mutant is a nongerminating, extreme dwarf, which is phenotypically similar to strong alleles of the *ga1* and *ga3* mutants. (Koornneef and van der Veen, 1980). The biochemical characterization of the *ga2* mutant was described by Zeevaart and Talon (1992). The *ga2* mutant is responsive to exogenous *ent*-kaurene and is lacking in the ability to accumulate *ent*-kaurene when treated with an inhibitor of *ent*-kaurene metabolism. A cell-free extract prepared from siliques of the *ga2* mutant lacked KS activity. Therefore, the *GA2* locus probably encodes either KS or a regulatory protein controlling the expression of the *A. thaliana* KS gene, *AtKS*. Several GA-responsive dwarf mutants from other plant species also contain reduced KS activity. Hedden and Phinney (1979) reported that the formation of *ent*-kaurene from mevalonic acid, GGDP, or CDP is reduced in cell-free preparations from the maize (*Zea mays*) *dwarf-5* mutant. Studies using cell-free extracts suggested that the *gib-3* mutant of tomato (*Lycopersicon esculentum*) is impaired in KS activity, leading to the GA-deficient mutant phenotype (Bensen and Zeevaart, 1990).

We previously purified KS from pumpkin (*Cucurbita maxima*; Saito et al., 1995) and isolated the corresponding cDNA (Yamaguchi et al., 1996). In this paper we report the isolation of the *AtKS* gene that we used to examine whether the *GA2* locus encodes KS and to study the regulation of genes encoding the interacting enzymes CPS and KS in *A. thaliana*. Using the *C. maxima* KS (*CmKS*) cDNA, we cloned

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Abbreviations: BAC, bacterial artificial chromosome; CDP, copalyl diphosphate; CPS, copalyl diphosphate synthase; GGDP, geranylgeranyl diphosphate; KS, *ent*-kaurene synthase; RACE, rapid amplification of cDNA ends; TEAS, tobacco 5-*epi*-aristolochene synthase; YAC, yeast artificial chromosome.

a homologous cDNA from *A. thaliana* and demonstrated that it encodes KS. Expression of the *CmKS* cDNA in *ga2–1* complemented the mutant phenotype. We also demonstrated that the *AtKS* cDNA from the *ga2–1* encodes a truncated protein that does not have any KS activity in an in vitro enzyme assay.

MATERIALS AND METHODS

Arabidopsis thaliana (L.) Heynh. (ecotype Lansberg *erecta*) plants were used in this study. The *ga2–1* mutant seeds were obtained from the Arabidopsis Biological Resource Center (The Ohio State University, Columbus). Before planting, the *ga2–1* seeds were incubated in 100 μM GA_3 solution at 4°C for 3 d. The plants were grown either on Murashige-Skoog agar medium (GIBCO-BRL) or in soil under 16-h light/8-h dark conditions at 22°C. The *ga2–1* mutant was sprayed with 100 μM GA_3 solution to produce seeds.

Complementation Test

To overexpress the *CmKS* cDNA, a gene fusion containing the cauliflower mosaic virus 35S-promoter with dual enhancers, the tobacco etch virus-nontranslated region, *CmKS* cDNA, and the nopaline synthase terminator was constructed in the binary vector pBI101.2 as follows. The nucleotide sequence around the first ATG codon of *CmKS* cDNA (Yamaguchi et al., 1996) was modified to contain an *Nco*I site, and an internal *Nco*I site near the 3′ end of the coding sequence was modified by PCR. Introduction of the *Nco*I site at the starting ATG converts the second codon TAT (Tyr) to GAT (Asp). Modification of the internal *Nco*I site does not alter the CmKS protein sequence. The forward (5′-ACCAGCCATGGATCTTTCCCGACCTACCGGCG; *Nco*I site is underlined) and reverse (5′-TTGAGCTCATTTGTTCAATAGTGCATCCAGATCCATAGGTT; *Sac*I site and the altered *Nco*I site are underlined) primers were used to amplify a 2.4-kb DNA fragment from pCmKB-1 (Yamaguchi et al., 1996). The 2.4-kb DNA fragment was inserted into the *Nco*I-*Sac*I site of pRTL2 (Restrepo et al., 1990). This plasmid was digested with *Hin*dIII and *Sac*I, and the resulting 3.1-kb DNA fragment was ligated into the *Hin*dIII-*Sac*I sites of the binary vector pBI101.2 (pBI/KSB101). Transgenic plants were generated by vacuum infiltration with *Agrobacterium tumefaciens* (Bechtold et al., 1993).

Isolation of Genomic and cDNA Clones

Plaque lifts from an *A. thaliana* (ecotype Columbia) genomic library (λDASHII; courtesy of Dr. M. Matsui, RIKEN Institute, Saitama, Japan) on nylon membranes (Hybond-N^+, Amersham) were hybridized with a radiolabeled *CmKS* cDNA (2.7 kb; Yamaguchi et al., 1996) at 55°C in Rapid-hyb buffer (Amersham). The membranes were washed with 2× SSC (Sambrook et al., 1989) containing 0.1% SDS at 60°C. One of the clones isolated (λAtKS-42) was further characterized. A 3.5-kb DNA fragment, which was generated by *Eco*RI digestion from the λAtKS-42, was subcloned into the *Eco*RI site of pUC118 (Toyobo, Osaka, Japan) and was named pgAtKS1.

Isolation of the corresponding cDNA fragments was performed by using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA), according to the manufacturer's protocol. Based on the DNA sequence of pgAtKS1, oligonucleotide primers AtKS1F (5′-TGAGCAAACAAAGGAGAAGATTAGG), AtKS1R (5′-CCTAATCTTCTCCTTTGTTTGCTCA), and AtKS2R (5′-CCCAACTAGTATCGTAGGCCG) were synthesized. Poly(A^+) RNA was prepared from 14-d-old wild-type plants using a magnetic resin (PolyATtract mRNA isolation systems, Promega). For 5′ RACE, a reverse-transcription reaction was primed with the AtKS2R primer instead of the oligo(dT) primer. A cDNA template for 3′ RACE was prepared according to the Clontech manual. PCR was carried out using the gene-specific primers AtKS1R and AtKS1F (for 5′ and 3′ RACE, respectively) and the Expand High Fidelity PCR System (Boehringer Mannheim). The RACE products were subcloned into the pCRII vector (Invitrogen, San Diego, CA).

The DNA sequence of several independent clones (about 0.2 kb) from the 5′ RACE product was determined. Clones from the 3′ RACE (approximately 2.3 kb) were partially sequenced from both ends. The sequences from the RACE products were used to design a pair of primers 5′*Bam*HI/AtKS (5′-TTGGATCCGTTGCTACGACGCCGTTTCGG) and 3′*Sal*I/AtKS (5′-TGTCGACCTCTTCTTGTTCTGAAGCAAC) to amplify a 2.5-kb cDNA from the cDNA pool, which was prepared using 14-d-old *A. thaliana* mRNA. The PCR product was digested with *Bam*HI and *Sal*I and inserted into the *Bam*HI-*Sal*I site of pET28c vector (Novagen, Madison, WI) to generate pET/AtKS. The 2.5-kb cDNA from pET/AtKS was subcloned into pBluescript SK+ (pAtKS4) to determine the complete nucleotide sequence.

Heterologous Expression in *Escherichia coli*

A His-T7-tag-AtKS fusion protein was produced in *E. coli* BL21 (DE3) containing pET/AtKS. Expression of the fusion protein was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside when the *E. coli* cells reached an A_{600} of 1.0 at 37°C. After the addition of isopropyl β-D-thiogalactopyranoside, the bacteria were cultured at 20°C for 16 h. To detect the fusion protein by immunoblot analysis, total cell extracts were fractionated on an 8% SDS-polyacrylamide gel (Sambrook et al., 1989).

Isolation and overexpression of the *AtKS* cDNA from the *ga2–1* mutant was performed as described above. The corresponding plasmid construct was named pET/ga2. To rule out the possibility that errors in the sequence were generated during PCR, we repeated these experiments using several pET/AtKS and pET/ga2 clones that were generated from independent PCRs.

Enzyme Assay of the Fusion Protein

KS activity in *E. coli* extracts was determined as described previously (Yamaguchi et al., 1996) using [^{3}H]CDP and soluble crude extracts containing 40 μg of protein. Identification of *ent*-kaurene by full-scan GC-MS was per-

formed using unlabeled GGDP as a substrate according to the methods of Ait-Ali et al. (1997).

DNA Sequencing

DNA sequences were determined using a DNA sequencer (model ABI373 or ABI377, Applied Biosystems). A series of deletion clones was generated from the genomic clone pgAtKS1 and the cDNA clone pAtKS4 using Exonuclease III and S1 nuclease (GIBCO-BRL). To sequence the *AtKS* cDNA from the *ga2–1* mutant, cDNA fragments were amplified by two independent PCRs using double-stranded cDNA prepared from the *ga2–1* mutant as the template. The PCR products were mixed and directly sequenced using internal primers.

Immunoblot Analysis

An alkaline phosphatase-conjugated T7-tag antibody (Novagen), which recognizes the sequence MASMTGGNN adjacent to His-tag, was used to detect the His-T7-tag fusion protein according to the manufacturer's protocol.

DNA and RNA Gel-Blot Analysis

DNA gel-blot analysis was performed using the 2.5-kb *AtKS* cDNA (from pAtKS4) as a probe. The membrane was hybridized at 55°C in Rapid-hyb buffer (Amersham) and washed with 2× SSC containing 0.1% SDS at 60°C (low stringency). The membrane was then washed with 0.1× SSC containing 0.1% SDS at 65°C (high stringency).

Poly(A^+) RNAs were separated on a 1% agarose gel and blotted onto a nylon membrane. The 2.7-kb *CmKS* cDNA from pCmKB-1 (Yamaguchi et al., 1996) and the 2.5-kb *AtKS* cDNA were radiolabeled and used as probes to detect KS transcripts from pumpkin (*Cucurbita maxima*) and *A. thaliana*, respectively. For a loading control, the membranes were reprobed with radiolabeled cDNA for cytosolic cyclophilin (Lippuner et al., 1994). The membranes were exposed to a Storage Phosphor Screen and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Quantitation of radioactivity in each band was performed using IMAGEQUANT software (Molecular Dynamics).

Physical Mapping of the *AtKS* Gene

Hybridization data presented at the web site (http://cbil.humgen.upenn.edu/~atgc/physical-mapping/physmaps.html) were used to estimate the map position of the BAC clone F12J12 (accession no. B08170), which contained an identical DNA sequence to the *AtKS* gene. The F12J12 hybridized to BAC F19N14, which hybridized to BACs F16N6 and F17C14. These BACs were identified by hybridization to the YAC CIC1E4, which spans the bottom of chromosome I (Creusot et al., 1995) and contains the restriction fragment-length polymorphism marker m132 (Chang et al., 1988). Because all of these BACs also hybridized to YAC yUP8H12, which has been mapped at the top of chromosome I, YAC yUP8H12 is likely to be chimeric.

Sequence Comparison and Alignments

The BLAST (Altschul et al., 1990) program was used to search for homologous sequences in the database. The GAP and PILEUP programs in the Genetics Computer Group programs (University of Wisconsin, Madison) were used for comparison of amino acid sequences and generation of sequence alignments.

RESULTS

Complementation of the *ga2–1* Mutant

We used the *CmKS* cDNA to complement the mutant because the *ga2* mutant is deficient in KS activity. The *ga2–1* mutant was transformed with pBI/KSB101 via *A. tumefaciens*, and six independent kanamycin-resistant transgenic lines (T_1) were isolated. In four lines, the T_2 seeds segregated approximately 3:1 for kanamycin resistance versus sensitivity. The kanamycin-resistant seeds germinated in the absence of GA and grew identically to wild-type plants with normal fertility (Fig. 1A); conversely, the seeds that required GA for germination were kanamycin sensitive. These results demonstrated that expression of the *CmKS*

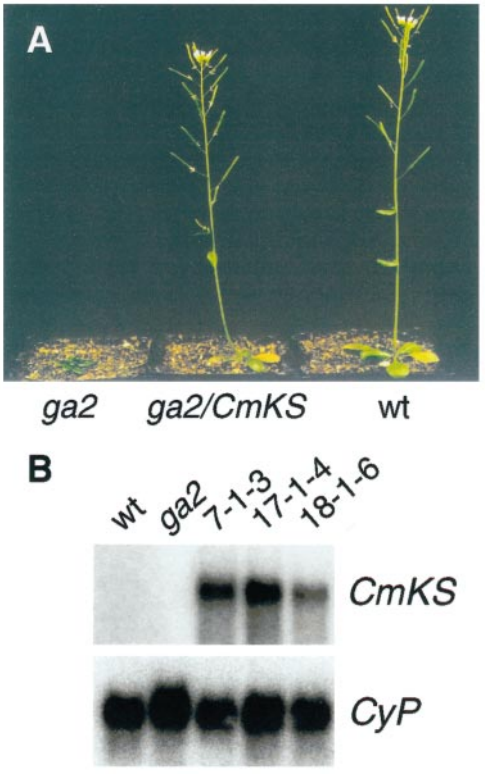

Figure 1. Expression of *CmKS* cDNA in the *ga2* mutant. A, The *ga2–1* mutant (*ga2,* an extreme dwarf), the *ga2–1* mutant expressing *CmKS* cDNA (*ga2/CmKS*), and a wild-type plant (wt). The plants were 35 d old. B, Autoradiogram of an RNA blot showing expression of the *CmKS* cDNA. Poly(A^+) RNAs (approximately 3 μg) isolated from wild type (wt), *ga2,* and the *ga2–1* mutant transformed with pBI/KSB101 (lines 7–1-3, 17–1-4, and 18–1-6) were probed with radiolabeled *CmKS* cDNA and then reprobed with a cyclophilin cDNA (*CyP*).

cDNA was able to rescue the phenotype of the *ga2* mutant. Three lines homozygous for the transgene (7–1–3, 17–1–4, and 18–1–6) were identified in the T_3 generation. Expression of the transgene was analyzed in the homozygotes by RNA blot analysis using *CmKS* cDNA as a probe (Fig. 1B). A 2.6-kb transcript was detected in RNA from the transgenic plants, whereas no bands were detectable in RNAs from wild-type and the *ga2* mutant. These data confirmed that the *CmKS* gene was expressed in the transgenic plants.

Isolation of *AtKS* Genomic and cDNA Clones

To determine whether the *GA2* locus encodes AtKS and to study the regulation of *AtKS* expression in *A. thaliana*, we set out to isolate the *AtKS* gene. We first carried out DNA blot analysis of *A. thaliana* genomic DNA using the *CmKS* cDNA as a heterologous probe to determine the appropriate condition for cloning the *AtKS* gene (data not shown). Under the same low-stringency hybridization conditions, an *A. thaliana* genomic library was screened and two positive clones were isolated from 2×10^5 plaques. Restriction enzyme maps of these clones suggested that they were derived from a single locus. A 3.5-kb DNA fragment derived from one of the λ clones was subcloned into a plasmid vector (pgAtKS1; Fig. 2) and partially sequenced (data not shown). The predicted amino acid sequence showed significant sequence similarity to CmKS. Oligonucleotide primers based on the genomic DNA sequence were used to amplify 0.2- and 2.3-kb cDNA fragments by 5′ RACE and 3′ RACE, respectively (Fig. 2). Based on nucleotide sequence of the RACE products, a pair of primers was synthesized to amplify a 2.5-kb *AtKS* cDNA (Fig. 2; pAtKS4).

The nucleotide sequence of the full-length *AtKS* cDNA was determined (accession no. AF034774). An open reading frame consisting of 2355 nucleotides (785 amino acids) was observed (Fig. 3). The predicted molecular mass of the translated product is 90 kD, and the deduced amino acid sequence shows 70% similarity (52% identity) to CmKS.

Functional Studies of the AtKS Protein

To verify that the isolated gene encodes KS, the full-length cDNA was overexpressed in *E. coli* and the enzymatic activity of the recombinant AtKS protein was tested in vitro. The 2.5-kb *AtKS* cDNA was subcloned into the expression vector pET28c (pET/AtKS), and a recombinant protein was produced as a fusion with His-T7-tag (His-T7-AtKS). Immunoblot analysis was performed to confirm the production of the fusion protein using a T7-tag antibody (Fig. 4A). In extracts of *E. coli* carrying the pET/AtKS, an 88-kD protein (His-T7-AtKS) was recognized by the antibody. No bands were detected in lysates of *E. coli* carrying the control plasmid pET28c.

KS activity in extracts from *E. coli* was determined using [^{3}H]CDP as the substrate (Yamaguchi et al., 1996). A significant amount of [^{3}H]*ent*-kaurene (491 dpm) was detected when [^{3}H]CDP was incubated with the *E. coli* extracts containing His-T7-AtKS. In contrast, [^{3}H]*ent*-kaurene produced from the control extract of *E. coli* carrying pET28c was not above background level (23 dpm), indicating that the His-T7-AtKS possesses significant KS activity (Fig. 4B). The identity of the product was confirmed by GC-MS, using GGDP as a substrate in the presence of a recombinant CPS from pea (Ait-Ali et al., 1997; Kawaide et al., 1997; data not shown). *ent*-Kaurene was identified by full-scan GC-MS from an incubation mixture containing both CPS and His-T7-AtKS. *ent*-Kaurene was not detectable in the control reaction mixture containing CPS and extract of *E. coli* harboring pET28c. These data confirm that we had isolated a cDNA encoding KS from *A. thaliana*.

Identification of the *ga2–1* Mutation

Previous biochemical studies (Zeevaart and Talon, 1992) and the result of the complementation test suggested that the *GA2* locus could encode either KS or a regulator of *KS* gene expression. The possibility that the *AtKS* gene corresponds to the *GA2* locus was examined. RNA-blot analysis indicated that the size and level of the *AtKS* transcript in the *ga2–1* were comparable to those in wild-type plants (data not shown). From this observation, the *GA2* gene is unlikely to encode a protein that regulates the transcription of the *AtKS* gene.

The *AtKS* cDNA isolated from the *ga2–1* mutant was inserted into the *E. coli* expression vector pET28 (pET/ga2), and the recombinant fusion protein was produced. By immunoblot analysis, a 74-kD protein was detected in lysates of *E. coli* carrying pET/ga2, whereas the observed mass of His-T7-AtKS was 88 kD (Fig. 4A). KS activity in *E. coli* extracts containing the truncated recombinant protein from the *ga2–1* was as low as the control *E. coli* extract (Fig. 4B). DNA sequence analysis further revealed that a base substitution of C-2099 to T is present in the *AtKS* cDNA from the *ga2–1* mutant. This mutation changes a Gln-678 codon (CAA) to a stop (TAA, Fig. 3) codon. The calculated mass of the truncated protein from the *ga2–1* was 77 kD. This was consistent with the result of the immunoblot analysis (Fig. 4B), and we concluded that the *AtKS* gene corresponds to the *GA2* locus. Therefore, the *AtKS* gene will be referred to as the *GA2* gene.

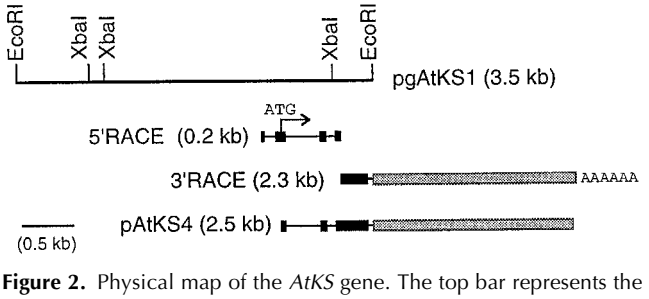

Figure 2. Physical map of the *AtKS* gene. The top bar represents the genomic clone pgAtKS1 with the *Xba*I restriction map. The RACE products and pAtKS4 (containing the coding region) are cDNA clones. Horizontal thin lines in the cDNA clones show introns and black boxes show exons. Gray bars indicate the stretches of cDNA where a corresponding genomic sequence was not obtained in this study. The position of the first ATG codon and the direction of the open reading frame are indicated by an arrow.

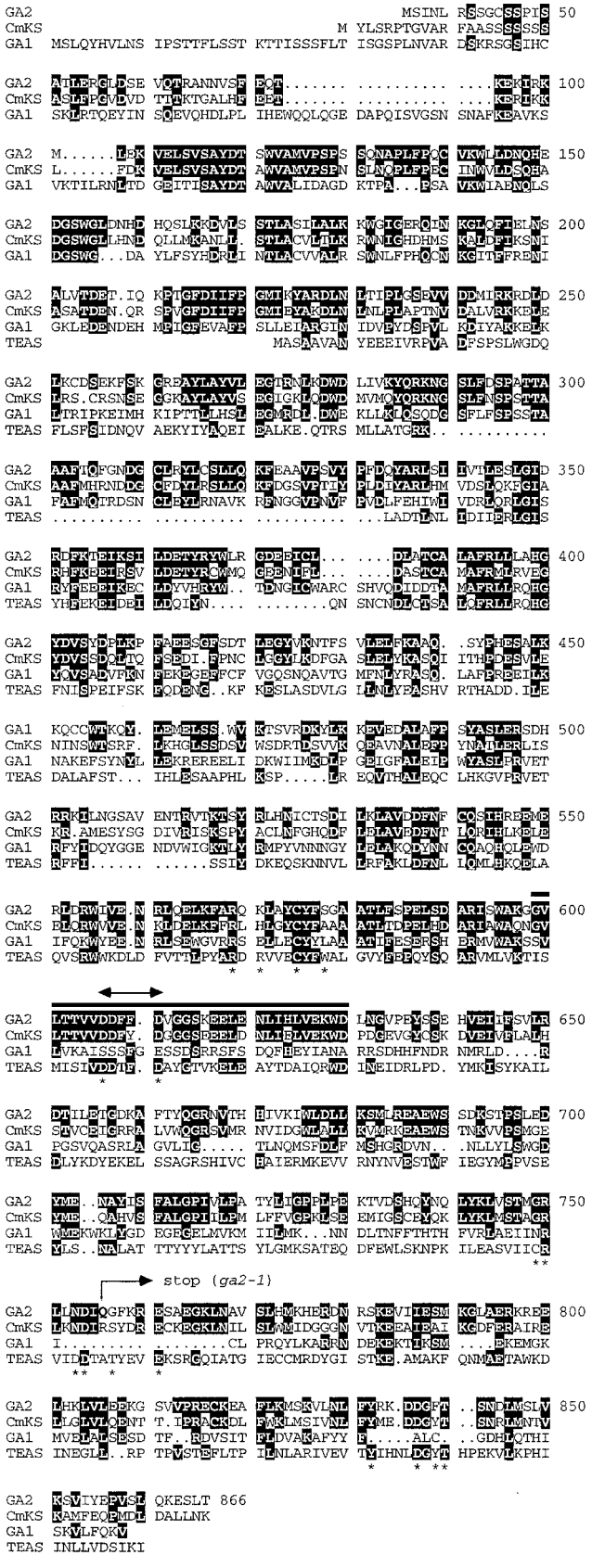

Figure 3. Sequence alignment of plant terpene cyclases. Deduced amino acid sequences of CmKS (Yamaguchi et al., 1996), GA1 (*A. thaliana* CPS; Sun and Kamiya, 1994), and TEAS from *Nicotiana tabacum* (Facchini and Chappell, 1992) were compared with that of AtKS (GA2). Amino acids conserved between GA2 and at least one other cyclase are highlighted in reverse print. The amino acids that are proposed to be involved in the formation of the active site of TEAS (Starks et al., 1997) are indicated with asterisks below the sequence. The conserved DDXXD motif is indicated with a line with double arrowheads. The 32-amino acid stretch, which is highly conserved between GA2 and CmKS, is highlighted with a thick horizontal line. The position of the mutation in *ga2–1* (Q to a stop codon) is shown with an arrow. Dots are gaps for optimization of alignments.

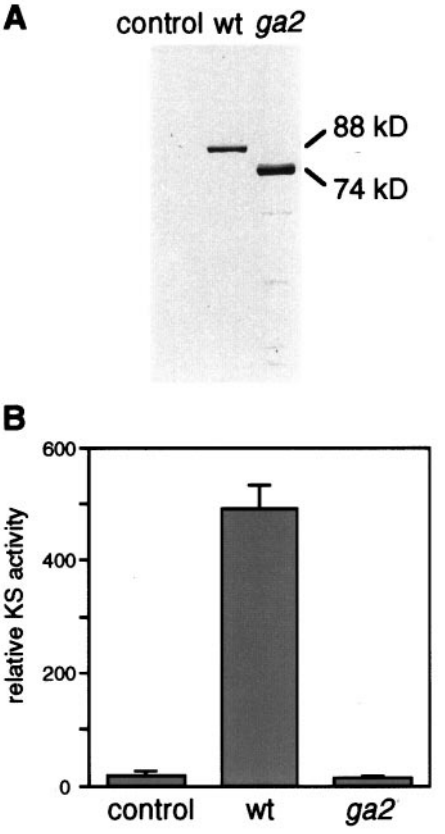

Figure 4. Functional studies of the *AtKS* gene from wild-type and the *ga2–1* mutant. A, Immunoblot containing total cell lysates from *E. coli* carrying pET28c (control), pET/AtKS (wt), and pET/ga2 (*ga2*). The membrane was probed with an alkaline phosphate-conjugated T7-tag antibody. The observed molecular mass of each band is shown on the right. B, KS activities of soluble protein extracts (40 μg of protein each) prepared from *E. coli* indicated in A. Results are means ± SE.

A database search revealed that a BAC end sequence contains a sequence identical to *GA2*. Because the *GA2* gene appeared to be a single-copy gene in the haploid genome (see below), the BAC is most likely to contain the *GA2* locus. According to hybridization data (http://cbil.humgen.upenn.edu/~atgc/physical-mapping/physmaps.html), the BAC clone is located at the bottom of chromosome I. These data coincide with the genetic mapping data of the *GA2* locus to the same region of chromosome I (Koornneef

and van der Veen, 1980), supporting the conclusion that we have isolated the *GA2* locus.

DNA and RNA Blot Analysis

To examine the presence of *GA2* homologs in *A. thaliana*, genomic DNA blot analyses were carried out using the *GA2* cDNA as a probe under low- and high-stringency conditions. The result of the experiment under low-stringency conditions is shown in Figure 5A, and an identical banding pattern was observed under high-stringency conditions (data not shown). These results suggest that no closely related sequences exist in *A. thaliana*.

Tissue distribution of the *GA2* transcripts was studied by RNA blot analysis (Fig. 5B). The *GA2* mRNA was detected in all tissues that we examined. A cDNA for cytosolic cyclophilin (Lippuner et al., 1994) was used as a probe to standardize RNA loadings. The mRNA level of *GA2* was approximately 4 times higher in young siliques than in cauline leaves in 35-d-old plants.

Primary Structure of GA2

The predicted amino acid sequence of GA2 showed significant homology to other terpene cyclases with enzymatic activities distinct from KS. These cyclases include GA1 (*A. thaliana* CPS; 55% similarity, 31% identity; Sun and Kamiya, 1994), abietadiene synthase from *Abies grandis* (55% similarity, 34% identity; Vogel et al., 1996), taxadiene synthase from *Taxus brevifolia* (54% similarity, 32% identity; Wildung and Croteau, 1996), and TEAS (50% similarity, 25% identity; Facchini and Chappell, 1992). The sequence alignment of GA2, CmKS, GA1, and TEAS is shown in Figure 3.

A putative transit peptide sequence is present in the N terminus of GA2, as was also observed in the CmKS (Yamaguchi et al., 1996). The first 44 amino acids are rich in hydroxylated amino acids (25% are Ser or Thr), and the predicted pI of this stretch is 10.3, which agrees with the common characteristics of transit peptides that target proteins into plastids (Keegstra et al., 1989).

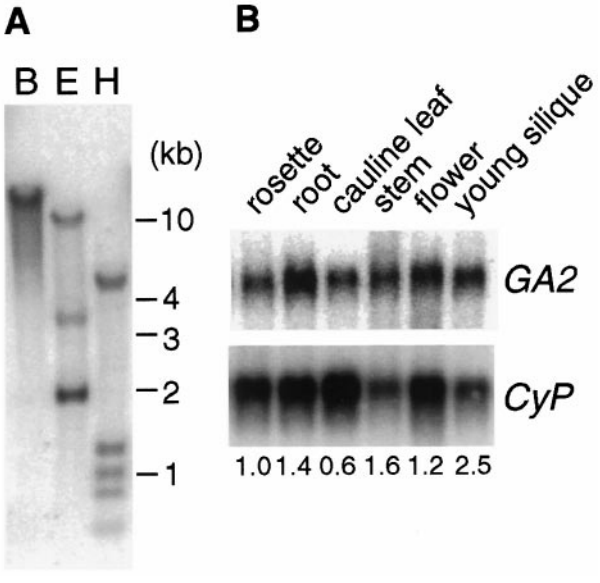

Figure 5. Genomic DNA and RNA blot analyses of the *GA2* gene. A, Autoradiogram of a DNA blot containing *A. thaliana* genomic DNA digested with *Bam*HI (B), *Eco*RI (E), or *Hin*dIII (H). Hybridization was carried out under low-stringency conditions using radiolabeled *GA2* cDNA as a probe. B, Autoradiogram of an RNA blot containing poly(A^+) RNAs purified from 300 μg of total RNAs isolated from different tissues. Aerial tissues (rosette) and roots were harvested from 14-d-old plants grown on agar medium. Cauline leaves, main stems (stem), flower clusters, and young siliques (which contain globular or heart-shaped embryos) were harvested from 35-d-old plants on soil. The membrane was probed with radiolabeled *GA2* cDNA and then stripped and reprobed with radiolabeled cyclophilin cDNA (*CyP*). Numbers below the blot indicate the relative amount of *GA2* mRNA standardized by the relative levels of cyclophilin mRNA ("rosette" was arbitrarily set as 1.0).

DISCUSSION

Our results show that expression of the *CmKS* cDNA was able to complement the *A. thaliana ga2* mutant. We isolated and characterized an *A. thaliana* cDNA encoding KS and demonstrated that the KS cDNA from the *ga2–1* mutant contains a nonsense mutation. These data allow us to conclude that the *GA2* gene encodes KS and confirm that a mutation in KS causes the GA-deficient phenotype of the *ga2* mutant.

To complement the *ga2* mutant, the *CmKS* cDNA was expressed under the regulation of the cauliflower mosaic virus 35S promoter with dual enhancers and tobacco etch virus-nontranslated region, which often results in overexpression (Carrington and Freed, 1990; Sun and Kamiya, 1994). Overproduction of the KS protein could increase GA biosynthesis and, consequently, result in a phenotype that is similar to wild-type plants treated with an excess amount of GAs. However, phenotypes of the four transgenic lines were almost indistinguishable from the wild-type plants. Immunoblot analysis using a specific antibody to the CmKS suggested that the levels of CmKS in the transgenic plants were much lower than in the *C. maxima* endosperm (data not shown), which contains an exceptionally high level of GA biosynthetic activity (Graebe, 1987). We have not determined the level of heterologous CmKS in the transgenic *ga2* mutants in comparison with the endogenous AtKS in wild-type plants. However, the CmKS may not have been overproduced to such an extent that it increases the active GA concentration above wild-type level. Alternatively, a higher level of KS protein may not significantly increase the amount of active GAs if other enzymes in the GA biosynthetic pathway are limited, or genes for those enzymes are regulated to control the level of active GAs. In fact, several genes encoding enzymes catalyzing later steps of GA biosynthesis are negatively regulated by GA activity (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995).

Comparison of the two KS proteins from *C. maxima* and *A. thaliana* indicates highly conserved amino acid stretches. One stretch of 32 amino acids (amino acid 599–630 in Fig. 3) contains the DDXXD motif, a putative binding site for the metal ion-diphosphate complex (Chappell, 1995; Mc-

Garvey and Croteau, 1995). Recently, the x-ray crystal structure of TEAS was reported (Starks et al., 1997). TEAS catalyzes the conversion of prenyl diphosphate to a cyclic hydrocarbon and contains the DDXXD motif. The x-ray structures with substrate analogs indicated that, in fact, the D residues of the DDXXD motif bind directly to the Mg^{2+}-diphosphate complex. It is likely that the DDXXD motif in KS is also involved in binding to the diphosphate group of the substrate.

The sequence data showed that the *ga2–1* allele has a single-base substitution that introduces a premature stop codon at position 756 (Fig. 3). Thus, the GA2 protein from the *ga2–1* mutant is missing 108 amino acids at the C terminus. The x-ray crystal structure of TEAS indicated that, in addition to the DDXXD motif, several other amino acid residues at the C terminus are also involved in binding to the substrate, including aromatic amino acid residues at positions 832 and 839 and E-761, D-837, and T-840 near the C terminus (Fig. 3; Starks et al., 1997). These amino acid residues are conserved in both KS proteins but are missing in the truncated protein in the *ga2–1* mutant. The absence of these amino acid residues and a possible conformational change resulting from the truncation of 108 amino acids could be the cause of the loss of enzymatic activity.

The *ga2–1* mutant still contains low levels of GAs (Zeevaart and Talon, 1992), although the recombinant AtKS protein from the *ga2–1* mutant was not functional in vitro and, presumably, not in vivo. The presence of GAs in the *ga2–1* mutant implies the existence of another enzyme that can catalyze the same reaction. However, we were unable to detect any *GA2* homologs by DNA blot analysis under low-stringency conditions using the *GA2* cDNA as a probe. Under the same hybridization conditions, the *CmKS* cDNA, which has 61% nucleotide sequence identity to the *GA2* cDNA, was able to detect the *GA2* DNA. Therefore, the cyclase that exhibits KS activity in the *ga2–1* mutant would have lower sequence similarity than CmKS.

The *ga2–1* mutant is a severe GA-deficient mutant with a phenotype that is a nongerminating, male-sterile, extreme dwarf (Koornneef and van der Veen, 1980). The phenotype suggests that the *GA2* gene may be responsible for the formation of *ent*-kaurene throughout the life cycle of the plant. The *GA2* transcripts were detected in every tissue examined in this study (Fig. 5B). From biochemical studies of CPS and KS activities in *Marah macrocarpus*, it was proposed that CPS and KS might interact to efficiently catalyze the two-step cyclization reaction (Duncan and West, 1981). Therefore, expression of genes encoding CPS and KS may be similarly regulated during plant development. Expression of the *GA1* gene is limited to specific cell types, even though the *GA1* transcripts are present in all organs (Silverstone et al., 1997), in which the *GA2* mRNAs were detected in this study. In addition, the *GA1* transcript levels were high in young siliques and were very low in cauline leaves (Silverstone et al., 1997). This tendency was also observed in the relative levels of the *GA2* mRNA (Fig. 5B). However, the *GA2* mRNA levels did not change as much as the *GA1* mRNA levels (Fig. 5B; Silverstone et al., 1997). In chloroplast lysates from apical shoots of pea, significant KS activity was detected, but CPS activity was absent in the same lysate (Railton et al., 1984). These results imply that CPS and KS may not necessarily be present at the same level during plant development. Further expression studies of the *GA2* gene using reporter genes or in situ RNA hybridization will reveal whether expression of the *GA2* gene is limited to the same cells as it is in the *GA1* gene.

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