

Cloning and Characterization of the Maize *An1* Gene

Robert J. Bensen,^a Gurmukh S. Johal,^{a,1} Virginia C. Crane,^a John T. Tossberg,^a Patrick S. Schnable,^b Robert B. Meeley,^a and Steven P. Briggs^{a,2}

^a Pioneer Hi-Bred International, Inc., P.O. Box 1004, Johnston, Iowa 50131

^b Department of Agronomy, Iowa State University, Ames, Iowa 50011

The *Anther ear1* (*An1*) gene product is involved in the synthesis of *ent*-kaurene, the first tetracyclic intermediate in the gibberellin (GA) biosynthetic pathway. Mutations causing the loss of *An1* function result in a GA-responsive phenotype that includes reduced plant height, delayed maturity, and development of perfect flowers on normally pistillate ears. The *an1::Mu2-891339* allele was recovered from a *Mutator* (*Mu*) F₂ family. Using *Mu* elements as molecular probes, an *An1*-containing restriction fragment was identified and cloned. The identity of the cloned gene as *An1* was confirmed by using a reverse genetics screen for maize families that contain a *Mu* element inserted into the cloned gene and then by demonstrating that the insertion causes an *an1* phenotype. The predicted amino acid sequence of the *An1* cDNA shares homology with plant cyclases and contains a basic N-terminal sequence that may target the *An1* gene product to the chloroplast. The sequence is consistent with the predicted subcellular localization of AN1 in the chloroplast and with its biochemical role in the cyclization of geranylgeranyl pyrophosphate, a 20-carbon isoprenoid, to *ent*-kaurene. The semidwarfed stature of *an1* mutants is in contrast with the more severely dwarfed stature of GA-responsive mutants at other loci in maize and may be caused by redundancy in this step of the GA biosynthetic pathway. DNA gel blot analysis indicated that *An1* is a single-copy gene that lies entirely within the deletion of the *an1-bz2-6923* mutant. However, homozygous deletion mutants accumulated *ent*-kaurene to 20% of the wild-type level, suggesting that the function of *An1* is supplemented by an additional activity.

INTRODUCTION

The morphological consequences of gibberellin (GA) deficiencies vary among plant genera but typically include reduced cell elongation and aberrant floral development (Reid, 1986). The phenotype of GA-responsive mutants of maize includes reduced plant stature due to shorter internode lengths, shorter broader leaves, and a reduced number of branches in the tassels. In addition, anthers develop on the pistillate ear, resulting in a perfect flower in the normally pistillate distal floret and a staminate flower in the normally aborted proximal floret of each spikelet on the ear (Emerson and Emerson, 1922). These phenotypes are illustrated in Figures 1A to 1D.

GAs are synthesized from the 20-carbon isoprenoid geranylgeranyl pyrophosphate (GGPP), beginning with the cyclization of GGPP to copalyl pyrophosphate (CPP) and then of CPP to *ent*-kaurene. This two-step process is catalyzed by kaurene synthases A and B (previously kaurene synthetases A and B), respectively (Duncan and West, 1981). In maize, and likely in most higher plants, *ent*-kaurene is oxidized stepwise to *ent*-7 β -hydroxykaurenoic acid, which is converted to the first GA in the pathway, GA₁₂-aldehyde (Suzuki et al., 1992). This is then oxidized to an active GA by one of three parallel pathways.

In maize, the major pathway appears to be the early 13-hydroxylation pathway (Hedden et al., 1982), with GA₁ being the primary bioactive product (Phinney and Spray, 1982).

The biosynthetic block in two of the GA-responsive mutants of maize, *d1* and *d5*, has been assigned by measuring accumulation of endogenous GA-biosynthetic intermediates and by observing growth responses to and metabolic fates of applied intermediates (Hedden and Phinney, 1979; Phinney and Spray, 1982; Spray et al., 1984; Fujioka et al., 1988). The biosynthetic role of a third gene, *Anther ear1* (*An1*) is less well defined. Mutations in *An1* result in a GA-responsive phenotype that is reversible with applied *ent*-kaurene (Katsumi, 1964), suggesting that the *An1* gene product functions in *ent*-kaurene synthesis.

Cloning and characterization of *An1* may clarify its function. The *Mutator* (*Mu*) transposable element system has been used to clone many genes based only upon the phenotype of a mutant allele (Walbot, 1992). One drawback of transposon tagging with *Mu* is that revertant alleles are rare. Therefore, confirming that a tagged gene has been isolated typically requires characterization of additional alleles. We have addressed this drawback by using a reverse genetics technology that permits the rapid recovery of new alleles containing *Mu* insertions. For *An1*, this was accomplished by using a pair of polymerase chain reaction (PCR) primers, one from the terminal inverted repeat

¹ Current address: Department of Agronomy, University of Missouri, Columbia, MO 65211.

² To whom correspondence should be addressed.

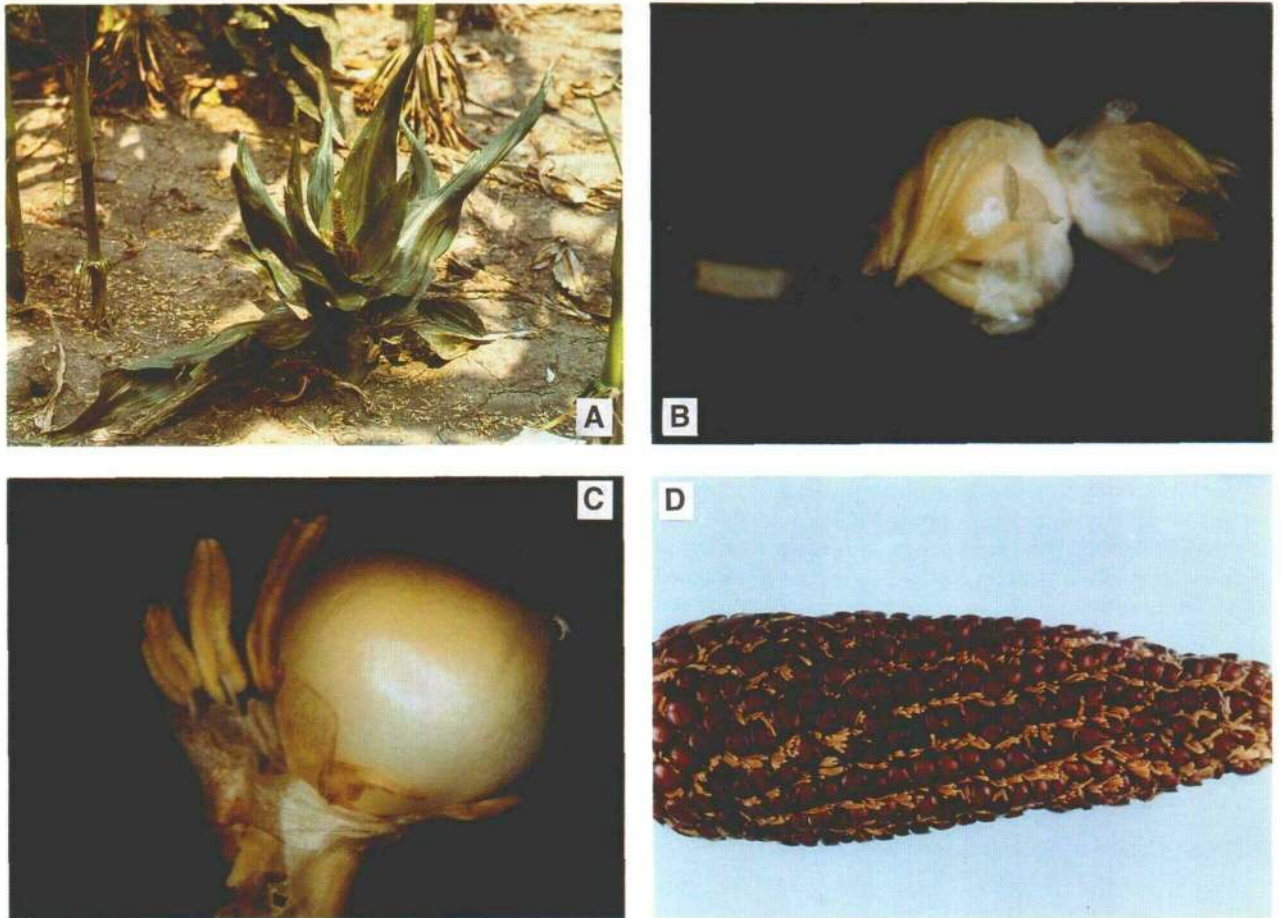


Figure 1. Phenotypes of GA-Responsive Maize Plants.

(A) Homozygous *an1::Mu2-891339* plant at maturity. Note the reduced internode lengths, foreshortened broad leaves, and unbranched tassel common to GA-deficient mutants of maize.

(B) Ear of *an1* plant at silking. Anthers are present in both florets of each spikelet of the ear.

(C) Ear spikelet of *an1* plant during grain filling. Anthers are in the proximal (left) and distal (right) florets. Note the full development of anthers in both florets, with the distal floret anthers spatially restricted by the developing kernel.

(D) Anthers on the ear of a mature *an1* plant.

of *Mu* and one from the putative *An1* clone, to survey a collection of DNA samples from individual plants for those that produce a PCR product with homology to the cloned gene. Such products are a consequence of *Mu* element insertion into the cloned gene. Seeds from plants whose DNA yielded such PCR products were planted and found to segregate for GA-responsive dwarfs, confirming that the cloned gene is *An1*.

RESULTS

Delay of Maturity in GA-Deficient Mutants

The *an1-bz2-6923* deletion allele of *an1*, when homozygous, did not cause a reduction in mature plant height or leaf length

when compared with wild-type siblings. Despite its similarity in final plant height, the mutant developed more slowly. In the example shown in Figure 2, this affect was manifest as a delayed pollen shed, which was on average a 200–heat unit delay or ~8 days. This observation indicates that time to maturity in maize is influenced more by the GA content of the plant than is vegetative development.

Partial Reversal of the Floral Morphogenic Program

All maize alleles of GA-responsive or GA-nonresponsive mutants share the phenotype of having anthers on the normally pistillate ear. Whereas pistil development in ears of GA-responsive mutants is normal, anther development is derepressed, with anthers developing in both florets of the ear. The result is perfect

flowers in the distal floret and staminate flowers in the proximal floret of each ear spikelet. Whereas the anthers of the distal floret are initially larger, the anthers in the proximal floret are eventually better developed (Figure 1C). The retardation of the distal anthers may be due to spatial constraints caused by the developing kernel. The proximal anthers produce mature pollen that accumulates starch and possesses a germ pore. Sexual whorl identity and development of tassel florets in these mutants are normal, with both florets developing fertile anthers, whereas pistillate structures fail to develop. The effect of these mutations in tassels appears to be limited to reducing branching and causing a poor pollen shed due to failure of the glumes to open and the filaments to elongate.

Role of GA in Gametophyte Competition

We tested the competitiveness of *an1* pollen against *An1* pollen using heterozygous *an1-bz2-6923* deletion mutant plants as the pollen source in a cross to homozygous *an1-bz2-6923* deletion mutant plants. The *Bronze2* (*Bz2*) gene provided a kernel marker to score the results of the cross, with bronzed kernels arising from pollinations by *an1* and purple kernels from *An1* pollen. Using homozygous *an1-bz2-6923* as the female parent minimized the potential for the silks to serve as a source of GA for the elongating pollen tube. The GA content of the silks appeared to be significantly reduced in homozygous *an1-bz2-6923* plants because the silks were delayed in their emergence from the ear husks and reduced in their final length. However, without actually measuring GA levels in silks, it cannot be ruled out that they provided sufficient GA to negate this competition test. Twelve crosses yielded 623 purple and 650 bronze kernels (a χ^2 value of 0.56 with a P value between 0.3 and 0.5). This result indicates that viable pollen ratios, germ

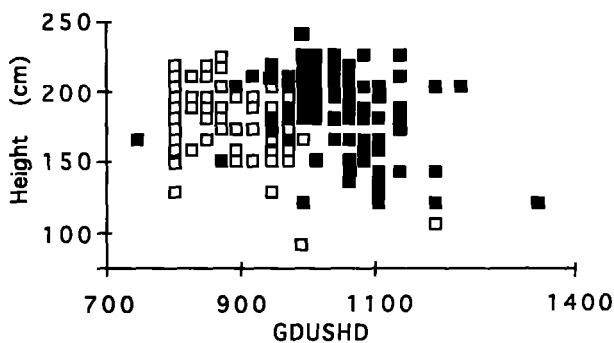


Figure 2. Role of GA in Maturity of Maize.

A comparison of heat units required to maturity for *an1-bz2-6923* and its wild-type siblings is shown. The height of wild-type siblings and *an1-bz2-6923* mutants at maturity is plotted versus growing degree units to pollen shed (GDUSHD). Under the growth conditions for this experiment, GDUSHDs accumulate at a rate of 20 to 25 per day. Thus, 200 GDUSHD is equal to 8 to 10 days. Each open square represents one wild-type sibling plant, and each closed square represents one *an1-bz2-6923* plant.

tube formation and extension, and fertilization of ovules are competitive between homozygous *an1* and *An1* pollen.

Cloning the *An1* Gene

A GA-responsive phenotype was observed to segregate as a simple recessive in an active *Mu* line. The mutation was shown to be allelic with *an1* and has been designated as *an1::Mu2-891339*. DNA gel blot analysis of genomic DNA from *an1::Mu2-891339* and its wild-type siblings identified a 5.4-kb restriction fragment that cosegregated with the mutation, as shown in Figure 3. This fragment was cloned into λ DNA. Restriction analysis of this clone identified fragments of flanking sequence DNA. A 2.6-kb *Xba*I flanking fragment was subcloned for use as a probe (g2.6Xba). DNA gel blot analysis, shown in Figure 4, of maize genomic DNA demonstrated that *An1* is a single-copy gene.

Selection and Sequence Analysis of *An1* cDNA

Using g2.6Xba as a probe, a 2.8-kb cDNA clone was recovered from a maize cDNA library. This cDNA appears to represent full-length mRNA based on RNA gel blot analysis; the primary product is a homologous transcript of \sim 2.8 kb, as shown in Figure 5. The cDNA contains an open reading frame of 2.5 kb or 823 amino acids, as illustrated in Figure 6. The predicted amino acid sequence shares significant homology with higher plant cyclase genes from mint (Colby et al., 1993), tobacco (Facchini and Chappell, 1992), and castor bean (Mau and West, 1994). The homology with the cyclases ranges from 20 to 25% identity and 45 to 53% similarity. The dicot cyclases use polyprenyl-pyrophosphates (PP-PPi) as substrates and contain a putative PP-PPi binding domain that includes the consensus sequence DDXXD. The predicted amino acid sequence of maize *An1* shares homology with the dicot cyclases on the immediate N-terminal side of and within the PP-PPi binding domain, but it lacks the final D residue, as illustrated in Figure 7A. In the maize *An1* gene product, beginning with the second X residue of the consensus sequence of the domain, there are 15 amino acids that share 67% identity and 93% similarity with the amino acid sequence of the tobacco cyclase and similar homologies with the other dicot cyclases. In the dicot cyclases, however, these 15 amino acids are not contiguous with the PP-PPi binding domain in the dicot cyclases but are found \sim 200 amino acids distal from the consensus sequence, toward the N terminus. Thus, relative to the dicot consensus, the PP-PPi binding domain in the maize *An1* gene is disrupted by a juxtaposition of this conserved 15-amino acid sequence.

The *An1* gene product contains a basic N terminus within which 12 of 32 amino acids are arginine and 17 of 32 are identical to a rice aspartate aminotransferase sequence (D16340), as illustrated in Figure 7B. The basic nature of the N terminus suggests that it functions in targeting the protein to the chloroplast (Keegstra et al., 1989). No putative ATP or GTP binding

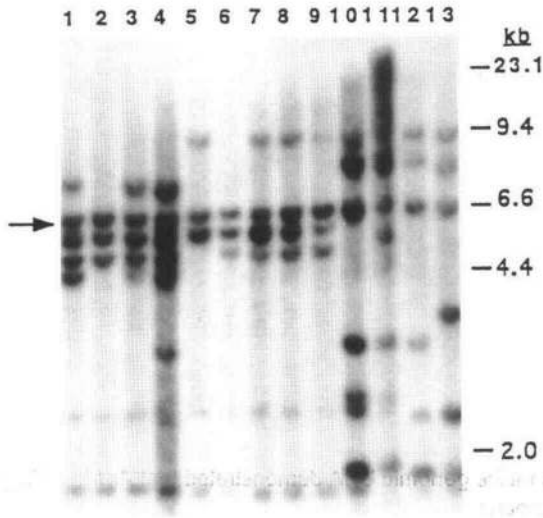


Figure 3. DNA Gel Blot Analysis: Cosegregation of the *an1::Mu2-891339* Allele and *Mu*-Containing Restriction Fragments.

Lanes 1 to 13 contain *Sst*I-restricted genomic DNA from 13 individual homozygous F_2 dwarfed *an1::Mu2-891339* siblings. The DNA gel blot was probed with an internal *Mu1* DNA fragment. A 5.4-kb restriction fragment partially homologous to *Mu1* and common to all *an1* individuals is identified with an arrow. Molecular length markers are shown at right in kilobases.

site consensus sequence (A/G-[X₄]-GK-S/T) is present in the predicted amino acid sequence of *An1*. This is consistent with *An1* encoding a synthase rather than a synthetase and supports the *in vitro* assay results, which suggest that no ATP or GTP is required for kaurene synthase activity (Duncan and West, 1981).

A comparison of cDNA and *an1::Mu2-891339* genomic DNA sequences demonstrated that a *Mu2* element is inserted within an exon and is 1.6 kb from the C terminus of the transcript.

Confirmation of Clone Identity

Independent *Mu*-induced *an1* alleles were used to confirm the identity of the putative *An1* clone. These independent alleles were obtained by a reverse genetics screen of a large collection of F_1 maize plants containing active *Mu* elements. The principle of the screen involves the pairing of a gene-specific primer with a *Mu*-specific primer to identify plants that contain *Mu* insertions in the target gene. A primer homologous to the terminal inverted repeat of *Mu* and two primers from the putative *An1* gene were used in such a screen. Two F_1 plants that yielded a PCR product hybridizing with the *An1* probe were identified. Shown in Figure 8A are the PCR products from DNA preparations of a homozygous wild-type control plant (lanes 1), the two heterozygous F_1 plants *an1::Mu-110H10* (lanes 2) and *an1::Mu-128E11* (lanes 3) that were selected for further

analysis, and a homozygous *an1::Mu2-891339* plant (lanes 4). A blot of the gel shown in Figure 8A was probed with a gene-specific probe from the putative *An1* clone. The results are illustrated in Figure 8B and confirm that the PCR products are homologous to the putative *An1* clone. As expected, when using the *Mu* terminal inverted repeat primer paired with either the reverse or forward primer from the putative *An1* clone, no products were formed from the wild-type plant DNA because no *Mu* elements were inserted in the putative *An1* gene of this plant. Conversely, these same primer pairs yielded PCR products from homozygous *an1::Mu2-891339* DNA and from heterozygous *an1::Mu-110H10* and *an1::Mu-128E11* plants, demonstrating that each of these three plants has a *Mu* element insertion in the putative *An1* gene.

All four plants yielded a PCR product of ~ 1.2 kb using just the forward and reverse primer pair. This product was expected from the wild-type plant and from the heterozygous *an1::Mu-110H10* and *an1::Mu-128E11* plants but not from homozygous *an1::Mu2-891339*. The primers were probably using the wild-type allele of the gene as the preferred template in the heterozygotes (F+R, lanes 2 and 3 of Figure 8A). The PCR product that was produced when using the forward and reverse primers on DNA from homozygous *an1::Mu2-891339* plants was unexpected because both copies of the putative *An1* gene have *Mu* element insertions. This PCR product may have resulted from the presence of revertant sectors (i.e., *Mu* excisions) or from synthesis that was facilitated by a looping out of the element. The sum of the sizes of the PCR products from the *Mu* primer paired with the forward and reverse primers was approximately equal

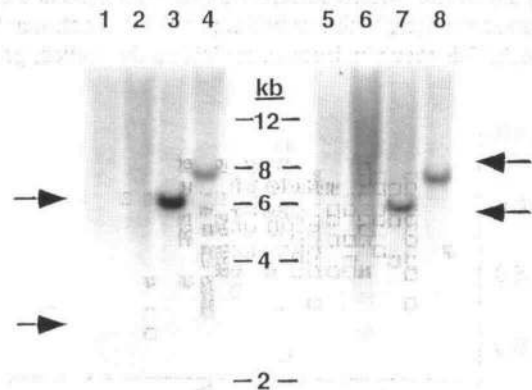


Figure 4. DNA Gel Blot Analysis: Deletion Mutant *an1-bz2-6923*.

A DNA gel blot of *Sst*I-digested genomic DNA of the deletion mutant (lanes 1, 2, 5, and 6), wild-type sibling DNA (lanes 3 and 7), and wheat DNA (lanes 4 and 8) was probed with a cDNA clone of *An1*. The amount of DNA applied to lanes 2 and 6 was twice that applied to the other lanes. Hybridization conditions for both blots were similar. Lanes 1 to 4 represent a high-stringency (65°C) wash, and lanes 5 to 8 represent a low-stringency (25°C) wash. Molecular length markers are shown between the blots in kilobases.

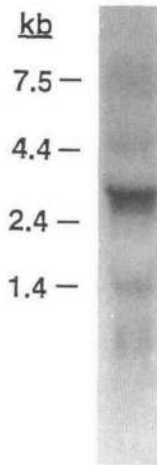


Figure 5. RNA Gel Blot Analysis: Total RNA Probed with the Genomic *An1* Subclone.

Ten micrograms of total RNA, prepared from shoots of light-grown maize seedlings, was used for RNA gel blot analysis. The g2.6Xba genomic subclone of *An1* was used to probe the blot. Molecular length markers are given at left in kilobases.

to the size of the product from the forward and reverse primers for all three mutant plants (lanes 2 to 4 of Figure 8A). The differences in the sizes of the individual PCR products that each of the three plants produced resulted from differences in the position of the *Mu* insertion relative to the primers and confirmed their independent derivation. Seed produced by self-pollination of the heterozygous *an1::Mu-110H10* and *an1::Mu-128E11* plants resulted in seedlings segregating for GA-responsive dwarfs. This demonstrated that both independently derived insertion alleles of the putative *An1* clone resulted in a GA-responsive, *an1* phenotype and confirmed that the cloned gene is *An1*.

Basis for Semidwarfed Nature of *an1* Plants

Unlike the other GA-responsive mutants of maize, *an1* is typically semidwarfed. The measurable growth response of *an1* plants to applied *ent*-kaurene suggests that it is deficient in the conversion of GGPP to *ent*-kaurene. Figure 4 shows a DNA gel blot of homozygous deletion mutant *an1-bz2-6923* DNA (lanes 1 and 2), tall sibling DNA (lane 3), and wheat DNA (lane 4) that was probed with the full-length *An1* cDNA and washed at high stringency. Hybridization to tall sibling DNA (indicated by the two arrows to the left of the figure) and to wheat DNA was detectable under these conditions, whereas no hybridization was detectable in lanes containing *an1-bz2-6923* DNA. This indicates that the *An1* gene lies within the deletion and that its transcript is not present in *an1-bz2-6923* plants. This mutant should therefore not be able to synthesize *ent*-kaurene. However, as shown in Table 1, light-grown *an1-*

bz2-6923 seedlings accumulated *ent*-kaurene in vivo at a reduced but significant level (20%) compared with their wild-type siblings. This observation was confirmed after application of tetcyclacis, an inhibitor of *ent*-kaurene oxidation. Tetcyclacis treatment also resulted in a similar difference in *ent*-kaurene levels between *an1::Mu2-891339* and its tall siblings. The approximately 10-fold increase in *ent*-kaurene levels observed in the tall siblings of both *an1* mutants reported in Table 1 was also observed in the commercial inbred line *B73*. The accumulation of *ent*-kaurene in the deletion mutant must have resulted from a non-*An1* activity that supplements *An1* production of *ent*-kaurene. When the stringency of the hybridization wash was lowered, hybridization to at least two *an1-bz2-6923* DNA restriction fragments was detectable in lanes 5 and 6 of Figure 4 (shown by the arrows to the right). This indicates that partially homologous, non-*An1* sequences are present in maize. Based on these observations, the semidwarfed phenotype of *an1* may have resulted from a partially homologous functionally equivalent gene. Such redundancy does not exist or is of little significance for maize *d5* mutants, which are severe dwarfs. The *d5* gene is thought to encode kaurene synthase B (Hedden and Phinney, 1979); its block of *ent*-kaurene synthesis appears to be nearly complete (Table 1).

DISCUSSION

Sequence Analysis

The predicted gene product of *An1* shares sequence homology with higher plant cyclase genes, including 5-*epi*-aristolochene synthase from tobacco (Facchini and Chappell, 1992), (-)-4S-limonene synthase from mint (Colby et al., 1993), and casbene synthase from castor bean (Mau and West, 1994). This is consistent with the role of the *An1* gene product as an enzyme catalyzing the cyclization of GGPP to *ent*-kaurene. The dicot cyclase activities used the polyprenyl-pyrophosphorylated compounds farnesyl, geranyl, and geranylgeranyl pyrophosphate, respectively, as substrates. The dicot cyclases are polypeptides of 50 to 60 kD that share 31 to 42% identity and 53 to 65% similarity with each other. They also share significant homology with the *An1* gene product. Their homology with each other is evenly distributed throughout their entire sequence as is their homology with *An1*. However, minimal homology exists between the *An1* gene product and the dicot cyclases in the N-terminal 150 to 250 amino acids of the *An1* gene product. This is most likely the result of *An1* encoding a larger polypeptide of ~83 kD with most of the nonhomologous coding sequence found at the 5' end of the transcript. The significance of the sequence homology remains unclear, except to suggest a cyclase function for the *An1* gene product.

The cloned higher plant cyclases act on monoterpenoids, sesquiterpenoids, and diterpenoids. Although their substrates differ, their proposed reaction mechanisms share the feature of an initial ionization of an allylic pyrophosphate (Hanson,

-102	CCGCTAGCTCTGCTTGTGTGCTGCTGATGCTGCGAGTTCCTCACCGGCTCTTTGCTT	-43	1278	ACCTCAACAGAGCCTCTCAGATAAGTTTTCAAGGAGAGGATGATTGCATCGTGCTAGGG	1337
-42	TTCTGCTTTTCACTGGCTGCGAGCTCAGCTGCTCAATCAGGTCCAAGCCGATCCGATC	17	1338	L N R A S Q I S F Q G E D V L H R A R V	1397
18	CGTATCCCTGGCAAGCAGCAGGAGGAGGAGGAGGCGCGCGCCGACGGGCGCCCGC	77	1398	TTTCTCTGTTAGTATTTCTGAGACAGAGAAACAAGGCATGATCCGTATAAATGGA	1457
78	Y P W Q S S R R R R R R R R R R R G R D G A P R	137	1458	TCGTTGCCAAGGATCTACCTGGCGAGGTCCAATATACACTAGACTTCCCTTGGTATGCA	1517
138	GGCAGCTCAGCTCGCCGGTGGTGGAGCGCGCAGCAGCGCCCGGCCACGCGACGA	197	1518	V A K D L P G E V Q Y T L D F P W Y A S	1577
198	Q P Q A R R V V E R A A A G P G H A T T	257	1578	GCTTGCCCTGCTAGAGGCAAGACCTATAGATCAATATGGTGGTAAAGTACGCTT	1637
258	CAACGCGAGCGCGCAACCTCTCCAGTGCCTAAAGTGTTCAGACCCGCTGTGAAA	317	1638	L P R V E A R T Y L D Q Y G G K D D V W	1697
318	T Q Q P D N V S S A K V F Q T S R V E T	377	1698	GGATTGGAAGACACTCTACAGGATGCCTCTTGAATAACGACACATATCTAGAGTTGG	1757
378	CGAGCTGAAATTCGAATTCGAGGAAACCAACAGACCTTGGAGTACGACCAAGCTC	437	1758	I C K T L Y R N P L V N N D T Y L E L A	1817
438	E S K L R N G R K R P Q D L E D E H Q A E	497	1818	CAATAGGGATTTCAACATTGCCAAGCTCGCATCAGCTTGAATGGGTGCAAAA	1877
498	AGGAGCGAGAGCTGACGCACTTATCGACAGGTGAGCGCATGCTACGCTCGATGAAC	557	1878	I R D F N H C Q A L H Q L E C N G L Q T	1937
558	E A E L Q P L I D Q V R A M L R S M N D	617	1938	CTGGTACAAAGATAATGGCTTGGCGCTTTTGGAGTAGAACCAAGATGTTTAAAGT	1997
618	ACGGGATACCGCGCTCCGGTACGACAGCGCTGGTGGCGATGCTCCGAAAGGTGG	677	1998	W Y K D N C L D A F G V E P Q D V L R S	2057
678	G D T S A S A Y D T A W V A M V P K V G	737	2058	CTTACTTTTAGCTGCTGTGCTATTTTGAACCTAGCCCTGCTAGAGCGCTGTGAT	2117
738	GGCGGAGCGCGCGCCAGCCCAAGTTCPPCCGACCGCTGGATCGGTGACACCC	797	2118	Y F L A A A C I F E P S R A E R L A W	2177
798	G D G G A Q P P Q P P P A T V R W I V D H Q	857	2178	GGCTAGAACCTCAATGATGGCAATGCCATTTCTACACATCTTCTGACATTTGGGAG	2237
858	AGCTGCCGACGGTCTCCGGGGGCGACTCGCCCTGTTCTCGCCTACGACCGCATCA	917	2238	A R T S M I A N A I S T H L R D I S E D	2297
918	L P D G S W G D S A L F S A Y D R M I N	977	2298	ACAAGAGAGATTGGAATGTTTCTGCACTGTCTCTATGAAGAAAACGATGATCATGGC	2357
978	ACACCCTCGCTCGCTGCTGCTGCTGACCAAGTGTCTGCGAGCCCGGAGGTCCGAGG	1037	2358	K K R L E C F V H C L Y E E N D V S W L	2417
1038	T L A C V V A L T K W S L E P A R C E A	1097	2418	TPAAGCAAAATCCATGATGTTTCTTGGAGGCGCATCGAAGATTAACTAATCTAT	2477
1098	CGGGCTCTCCTCTCGTACGAGAACATGTGAGGCTACGGGAGGAGCGCGGATGGA	1157	2478	K R N P N D V I L E R A L R R L I N L L	2537
1158	G L S F L H E N M W R L A E E E A E S M	1217	2538	1938	2597
1218	TGCCATCGGCTTGAGATCGCTTCCCTTCTCTCATCCAGACCGCTAGGACCTGGGG	1277	2658	1998	2657
	P I G F E I A F P S L I Q T A R D L G V			1998	
	TCGTCGACTTCCGCTACGACCAACCGCGCTGACGACATATACGCCAACAGGAGTCA			2058	
	V D F P Y G H P A L Q S I Y A N R E V K			2118	
	AGCTGAACGGATCCCAAGGACATGATGACAGGCTCCGACCTCCATCTGACAGCC			2178	
	L K R I P R D M M H R V P T S I L H S L			2238	
	TTGAAGGATGCTGACCTGGACTGGCGAGGCTTCTGAACCTCCAGTCCGACGGCT			2298	
	E G M P D L D W P R L L N L Q S C D G S			2358	
	CCCTCTGTTCTCTCCCTCCGCTACCGCTTACCGCTGATGCAAAACCGGTGACAGAGT			2418	
	F L F S P S A T A Y A L M Q T G D K K C			2478	
	GCTTCCGAATACATCGACAGGATGTCAAAAATTCAAACGGGAGTCCCAATGTTTATC			2538	
	F E Y I D R I V K K F N G G V P N V Y P			2598	
	CGTCTGATCTTTTCGACACACTTGGCTTCTGATCGGTTGAGCGGACTCCGATCTCC			2658	
	V D L F E H I W V V D R L E R L G I S R				
	GCTACTCCAACGAGAGATTGACAGTGTGATGACTATGTGAACAGGACTGGACTGAAG				
	Y F Q R E I E Q C M D Y V N R H W T E D				
	ATGGGATTTGCTGGCTACGAAATCCAAATGTGAAGGATCTGGATGACACAGCTATGGCT				
	G I C W A R K S N V K D V D D T A M A F				
	TCGACTACTAAGCTACNCTGACTACTCTCTCCAGTGTGTTTAAAGACCTTGTAGA				
	R L L R L H G Y N V S P S V F K N F E R				
	AAGATGGAGAGTTCITTTGTTTGTGGCCAACTGACTCAAGCCGTCACCTGGATGTATA				
	D G E F F C F V G Q S T Q A V T G M Y N				

Figure 6. Nucleotide and Predicted Amino Acid Sequences of *An1*.

The nucleotide and predicted amino acid sequences of *An1* are shown. The predicted amino acid sequence of the longest open reading frame is shown. The nucleotide sequence data reported here have been submitted to GenBank and assigned accession number L37750.

1972; Facchini and Chappell, 1992; Colby et al., 1993; Mau and West, 1994), resulting in the removal of the pyrophosphate moiety from the substrate as a part of their catalysis. A putative PP-PPi binding domain has been proposed to function in coordinating Mg^{2+} or Mn^{2+} cofactors via direct salt bridges to the metal ion that also complexes with the PPi group. The PP-PPi binding domain (I/L/V)XDDXXD is shared with enzymes such as farnesyl pyrophosphate synthase from *Escherichia coli*, yeast, rat, and humans and GGPP synthase from *Neurospora*, *Erwinia*, and *Rhodobacter*; all of these enzymes use pyrophosphorylated isoprenoids as substrates (for summary, see Math et al., 1992). The *An1* gene product lacks the final D residue of the PP-PPi binding domain consensus sequence. Instead, the *An1* gene product has juxtaposed at this position a 15-amino acid sequence that is found ~200 amino acids away in the dicots. For these 15 amino acids, the predicted amino acid sequence of the *An1* gene product is 67% identical and

93% similar to the dicots. The disruption of this binding domain may result in a loss in pyrophosphorylase function. This would be consistent with the possibility that *An1* encodes kaurene synthase A. Kaurene synthase A converts GGPP to CPP, which is a cyclization that occurs without the loss of the pyrophosphate moiety, whereas kaurene synthase B converts CPP to *ent*-kaurene, with the loss of the pyrophosphate (Shechter and West, 1969; Duncan and West, 1981).

The N terminus of the *An1* gene product has characteristics expected of a chloroplast targeting sequence (Keegstra et al., 1989), including a net positive charge (12 of 43 amino acids are basic; two are acidic). In addition, the *An1* N terminus also has a 53% identity with the N-terminal 27 amino acids of a rice aspartate aminotransferase clone (D16340). Aspartate aminotransferase has many isoforms, including some that are localized to the chloroplast (Wadsworth et al., 1993). The net positive charge and homology with the N terminus of the rice

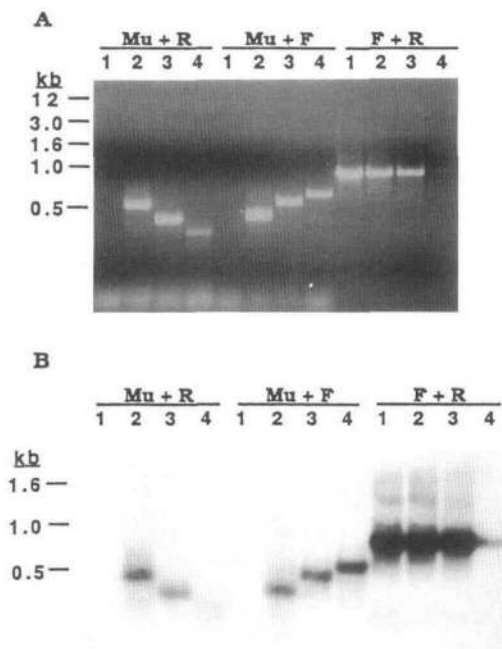


Figure 8. Confirmation of *An1* Clone Identity.

(A) Gene-specific PCR products. Gene-specific primers from the putative *An1* clone (F, forward primer; R, reverse primer) were paired with a *Mu* terminal inverted repeat primer (Mu + R and Mu + F) or each other (F + R). PCR-positive plants were identified from a large screen and shown individually as heterozygotes in lanes 2 (*an1::Mu-110H10*) and lanes 3 (*an1::Mu-128E11*). An inbred, nonmutated line (lanes 1) and homozygous *an1::Mu2-891339* (lanes 4) served as negative and positive controls, respectively.

(B) DNA gel blot of PCR products. A blot of the DNA gel shown in (A) was probed with a fragment of the putative *An1* clone (homologous to the sequence between the forward and reverse primers) to confirm the PCR product identity. Molecular length markers are indicated at left in kilobases.

METHODS

Plant Material

A *Mutator2* (*Mu2*)-tagged *anther ear1* (*an1*) maize allele, *an1::Mu2-891339*, was selected from lines with active *Mu* elements (lines were from D. Robertson, Iowa State University, Ames). Additional *an1* alleles used in this study include *an1-bz2-6923* (from M. Neuffer, University of Missouri, Columbia) and isolates *an1::Mu-110H10* and *an1::Mu-128E11* from the reverse genetics screen. *an1-bz2-6923* is a deletion mutant. The extent of the deletion has not yet been defined, although *id* (two map units proximal to *An1*) and *ad* (two map units distal from *Bz2*) are unaffected by the deletion.

DNA Gel Blot Analysis

Total DNA was extracted from leaf tissue by the urea extraction method (Dellaporta et al., 1983). DNA gel blot analysis was performed as

previously described (Johal and Briggs, 1992), using Duralose-UV membranes (Stratagene). A *Mu1* probe was synthesized by random priming (Amersham Corporation) a gel-eluted internal 650-bp *Ava*I-*Bst*II *Mu1* fragment isolated from pA/B5 (Chandler and Walbot, 1986). This internal *Mu1* fragment shares regions of homology with *Mu2* and permits hybridization to both *Mu1* and *Mu2* sequences.

Cloning Protocol

The genomic DNA restriction fragment containing the *Mu2* element of the *an1::Mu2-891339* allele was electroeluted after preparative agarose gel electrophoresis of *Sst*I-digested *an1::Mu2-891339* DNA, dialyzed, and concentrated by ethanol precipitation. Precipitated fragments were preannealed to *Sst*I-restricted arms of the bacteriophage vector λ *sep6/lac5* (from R. Martienssen, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and packaged using Gigapack Gold (Stratagene). This library was screened for *Mu*-containing phage, with the *Sst*I insert of a plaque-purified *Mu*-containing clone then transferred to the bacteriophage vector λ ZAPII (Stratagene). This insert and other clones used for probing or sequencing were all subcloned into pBluescript SK+ and maintained in SURE cells (Stratagene).

cDNA Library Screening

The maize cDNA library that served as a source for *An1* cDNA was prepared from whole kernels (30 days after pollination) of W22 (a gift from K. Cone, University of Missouri). Sequence data of a 2.8-kb *An1* cDNA were generated by Loftstrand Laboratories, Ltd., Gaithersburg, MD.

Table 1. *ent*-Kaurene Accumulation in Shoots of Light-Grown Maize Seedlings

Plant	<i>ent</i> -Kaurene Content (pmol/gfw)		Leaf Length (mm)	
	No Treatment	Tetacyclacis (48 hr)	2nd Leaf	3rd Leaf
<i>an1-bz2-6923</i>				
Tall	120	1330	42	83
Dwarf	33	209	30	58
<i>an1::Mu2-891339</i>				
Tall	61	710		
Dwarf	54	216		
<i>d5</i>				
Dwarf	ND	ND		
<i>B73</i>				
Tall	94	1093		

Seedlings were grown in continuous light for 6 days, at which time 20 μ M tetacyclacis (an inhibitor of kaurene metabolism) was applied directly to the shoots. Forty-eight hours later, the shoots of treated and untreated plants were analyzed for *ent*-kaurene content. gfw, grams fresh weight of tissue; ND, not detected. Tetacyclacis, an inhibitor of *ent*-kaurene oxidation, is manufactured by BASF, Ludwigshafen, Germany.

RNA Preparation and Gel Blot Analysis

Total RNA was prepared according to Chomczynski and Sacchi (1987). RNA gels were run, blotted, and probed as previously described (Johal and Briggs, 1992), using the g2.6Xba genomic flanking sequence subclone of *An1* to generate a random-primed probe.

Analysis of *ent*-Kaurene and Kaurene Synthase Activity

Analysis of the in vivo levels of *ent*-kaurene in light-grown maize seedlings was performed by J.A.D. Zeevaart, Michigan State University, East Lansing, as previously described (Zeevaart and Gage, 1993).

Polymerase Chain Reaction Analysis of *Mu*-Tagged *An1* Alleles

A large population of *Mu*-containing F₁ maize families (24,000 individuals) was tested by using polymerase chain reaction (PCR) to detect the presence of *Mu* insertional alleles in the *An1* gene. Primers specific for the putative *An1* clone were chosen from both sides of the *Mu* insertion in the *an1::Mu-891339* allele. A forward primer, 5'-GTG-TGGAAACCGAGTCCGAAATGCGAA-3', and a reverse primer, 5'-TAG-CCCAGCAAATCCCATCTCAGTCCA-3', were 788 and 404 bases from the *Mu2* insertion, respectively. These primers generated wild-type amplification products of ~1200 bp. The *Mu* primer 5'-CCCTGAGCT-CTTCGTC(CT)ATAATGGCAAT TATCTC-3' is partially homologous to the distal portion of the terminal inverted repeat common to all functional *Mu* elements. F₁ individuals containing insertions in the putative *An1* gene were identified by their production of PCR products with homology to the putative *An1* clone using either the forward or reverse primers paired with the *Mu*-specific primer. F₂ seed from plants producing PCR products were then planted in the greenhouse and scored for a gibberellin-responsive dwarf phenotype.

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