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Isolation of the Arabidopsis *GA4* Locus

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Progeny from a transgenic Arabidopsis plant generated by the Agrobacterium root transformation procedure were found to segregate for a gibberellin (GA)-responsive semidwarf phenotype. Complementation analysis with genetically characterized GA-responsive mutants revealed that the transgenic plant has an insertional mutation (*ga4-2*) that is an allele of the *ga4* locus. The semidwarf phenotype of *ga4-2* is inherited as a recessive mutation that cosegregates with both the T-DNA insert and the kanamycin resistance trait. DNA gel blot analysis indicated that the insertion site contains a complex T-DNA unit. A genomic library was constructed with DNA from the tagged *ga4* mutant; a DNA clone was isolated from the library that flanks the T-DNA insert. The plant sequence isolated from this clone was used to isolate the corresponding full-length genomic and cDNA clones from wild-type libraries. DNA sequence comparison of the clones to the existing data bases suggests that they encode a hydroxylase. This conclusion is in agreement with a biochemical study that indicated that the *ga4* mutant is deficient in 3β-hydroxylase in the GA biosynthetic pathway of Arabidopsis. RNA gel blot analysis showed that the message is ubiquitously expressed in different tissues of Arabidopsis but most abundantly in the silique. Unexpectedly, a higher level of transcription was detected in the ethyl methanesulfonate–induced *ga4* mutant, and this overexpression was repressed by treatment with exogenous GA.

INTRODUCTION

Gibberellins (GAs) are a large family of tetracyclic diterpenoid plant growth hormones that promote various growth and developmental processes in higher plants, such as seed germination, stem elongation, flowering, and fruiting (Crozier, 1983). A number of GA-responsive dwarf mutants have been isolated from various plant species, such as maize, pea, tomato, and Arabidopsis (Phinney, 1956; Koornneef, 1978; Koornneef et al., 1990; Reid and Ross, 1993). The dwarf mutants of maize (*dwarf-1*, *dwarf-2*, *dwarf-3*, and *dwarf-5*) were used to characterize the maize GA biosynthetic pathway by determining specific steps leading to biologically important metabolites (Phinney and Spray, 1982; Fujioka et al., 1988). Similar studies have been done with the dwarf mutants from pea (Reid and Ross, 1993). GA-deficient mutants have also been isolated from Arabidopsis (*ga1*, *ga2*, *ga3*, *ga4*, and *ga5*) (Koornneef and van der Veen, 1980). The Arabidopsis *ga4* mutant, induced by ethyl methanesulfonate (EMS) mutagenesis, is a germinating, GA-responsive, semidwarf whose phenotype can be restored to the wild type by repeated application of exogenous GA (Koornneef and van der Veen, 1980). In Arabidopsis, based on GA analysis, it appears that the *ga4* mutant allele blocks the conversion to 3β-hydroxy GAs, reducing the endogenous levels of GA_1, GA_8, and GA_4 and increasing the endogenous levels of GA_{19}, GA_{20}, and GA_9 (Talon et al., 1990). The reduced levels of the 3β-hydroxy GAs result in the semidwarf phenotype of the *ga4* mutant. The mutated *GA4* gene may therefore encode an altered form of 3β-hydroxylase, as has been suggested for the *le* mutant of pea (Ross et al., 1989).

We report here the identification of a new allele of the *ga4* locus generated by Agrobacterium root transformation (Valvekens et al., 1988). The mutant phenotype is inherited as a recessive mutation that cosegregates with the T-DNA insert, indicating that the *ga4* locus is tagged by T-DNA. In this study, we describe the cloning of the *GA4* locus and its transcriptional regulation.

RESULTS

Characterization of a T-DNA Insertion Mutant Allelic to *ga4*

In the course of plant transformation experiments designed for another purpose, a semidwarf mutant was generated from Arabidopsis (Landsberg *erecta* [*er*]) as a result of Agrobacterium-mediated root transformation (Valvekens et al., 1988). This mutant transgenic plant elongates its shoots in response to added GA_3 (Figure 1). There are several different GA-

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Figure 1. T-DNA–Tagged Mutant Is an Allele of the *GA4* Locus.

Both the T-DNA–tagged allele *ga4-2* (T) and the EMS-induced allele *ga4-1* (*ga4*) responded to GA_3 treatment with shoot elongation (T + GA_3 and *ga4* + GA_3, respectively). The complementation analysis of the *ga4-2* plant with *ga4-1* plant (*ga4* × T) revealed that the transgenic plant has an insertional mutation that is an allele of the *GA4* locus. W, canonical wild type, Landsberg *er.*

responsive mutants in Arabidopsis, and therefore to test for allelism, the transgenic plant was crossed to them in pairwise combinations. Complementation analysis with the other genetically characterized semidwarf mutants in Arabidopsis revealed that the cross between the transgenic plant and the EMS-induced *ga4* plant (Koornneef and van der Veen, 1980) does not complement the mutant phenotype (Figure 1). Therefore, the mutation in the transgenic plant is an allele of the *ga4* locus.

To test for cosegregation of the mutant phenotype and the T-DNA insert, the T_1 progeny of the transgenic mutant that exhibited the semidwarf trait were outcrossed either to an Arabidopsis *transparent testa2* (*tt2*) plant or to wild-type C24. The self-fertilized F_2 progeny from those two crosses were tested for segregation of the kanamycin resistance marker encoded by the T-DNA. Progeny were grown on sterile medium containing 50 mg/L kanamycin, and the ratio of kanamycin-resistant plants to kanamycin-sensitive plants was determined by their viability. Because approximately three-quarters of the F_2 progeny from both crosses were resistant to kanamycin (Table 1), the data indicate that there is one T-DNA insertion site in the transgenic plant. The self-fertilized F_2 progeny from the two crosses were also tested for segregation of the mutant phenotype. The results from both crosses (Table 2) show that a quarter of the resulting F_2 progeny exhibited the semidwarf phenotype, indicating that the semidwarf phenotype is inherited

Table 1. Segregation Ratios of the Kanamycin-Resistant and Kanamycin-Sensitive F_2 Progeny from *ga4-2* (T-DNA–Tagged Allele) Plants Crossed to *tt2* Plants or Crossed to C24 Wild-Type Plants

F_2 Plant	Kan^r:Kan^s	Approximate Segregation *Ratios*	T-DNA *Insertions*
ga4-2 × *tt2*	163:56	3:1	1 ($P > 0.8$)
ga4-2 × C24	104:29	3:1	1 ($P > 0.3$)

Progeny were grown on sterile mineral nutrient medium containing 50 mg/L kanamycin; the ratio of kanamycin-resistant (Kan^r) plants to sensitive (Kan^s) plants was determined from their viability. The number of T-DNA insertion sites predicted from the 3:1 segregation ratio and their probabilities from the chi-square test are shown.

Table 2. Segregation Ratios of the Wild-Type and Semidwarf F_2 Progeny from *ga4-2* (T-DNA–Tagged Allele) Plants Crossed to *tt2* Plants or Crossed to C24 Wild-Type Plants

F_2 Plant	Wild Type: Dwarf	Approximate Segregation Ratios	Mutant Loci
ga4-2 × *tt2*	151:53	3:1	1 ($P > 0.5$)
ga4-2 × C24	74:25	3:1	1 ($P > 0.9$)

Progeny were soil grown, and the ratio of plants that showed the wild-type compared with semidwarf phenotype was determined. The number of mutant loci predicted from the 3:1 segregation ratio and their probabilities from the chi-square test are shown.

as a single recessive mutation. Although the data from these two independent tests are indicative, we could not conclude that the *ga4* allele is tagged by the T-DNA insert. The presence of the insert and its linkage with the mutant trait were therefore further tested by DNA gel blot analysis.

Twenty F_3 progeny from self-fertilized F_2 plants (transgenic plant × *tt2*) were selected for their semidwarf phenotype and then tested for linkage of the T-DNA insert and the mutant phenotype by DNA gel blot analysis. DNA was isolated from leaf tissue of the individual F_3 progeny and digested with HindIII. After separation on an agarose gel and transfer to nylon filters, the DNA gel blot was probed with the ^{32}P-labeled pBIN19 plasmid containing the T-DNA border sequences (Bevan, 1984). The probe hybridized to DNA from all the representative transgenic plants, confirming the presence of the T-DNA insert (Figure 2). The hybridization pattern correlated with the T-DNA insert and the T-DNA–plant DNA junctions, and there was no hybridization with the wild-type (Landsberg *er*) control. The hybridization pattern also indicated that the insertion site contains a complex T-DNA unit (there are four fragments associated with the T-DNA insert in all representative plants) and that all of these fragments cosegregate with the mutant phenotype. Thus, data from both the segregation test (Table 2) and the DNA gel blot analysis (Figure 2) indicate that the T-DNA insert is the cause of the semidwarf mutation in the transgenic plant (the T-DNA–tagged allele will be referred to as *ga4-2*) and that the T-DNA insert is tightly linked to the *ga4* locus (the EMS-induced allele will be referred to as *ga4-1*).

Isolation of the *GA4* Gene

A genomic library was constructed with DNA isolated from F_4 progeny of the *ga4-2* plant. Genomic clone λT1-5 was derived by screening the genomic library with ^{32}P-labeled pBIN19 as a probe. After plaque purification, the clone was characterized by restriction enzyme analysis (Figure 3A). To identify the region that corresponds to the T-DNA insertion site, the HindIII fragments of the genomic clone were subcloned into the plasmid vector pBluescript KS–. The 1.2-kb HindIII fragment subclone pT12 contains the T-DNA–plant DNA junction and was used to identify the insertion site by sequencing into the T-DNA insertion break point. The plant sequences from the flanking 3.4-kb HindIII fragment subclone pT34 were used to isolate the corresponding wild-type genomic clone λWT6 (Figure 3B). The 3.2-kb HindIII subclone (pWT32) from λWT6 contains the sequences corresponding to the T-DNA insertion site in λT1-5 and was used as a probe to screen the wild-type leaf cDNA library and the *ga4-1* genomic library. The full-length *ga4-1* genomic and wild-type cDNA clones isolated span sequences contained in both clone pT34 and pWT32.

Nucleotide and Amino Acid Sequences

The *GA4* cDNA is 1077 nucleotides with an open reading frame of 359 amino acids (Figure 4). There is a single 433-bp intron whose position was deduced from a comparison of the cDNA and genomic sequences. Sequence analysis of the T-DNA–plant DNA junction indicates that the T-DNA insertion is within the intron. Analysis of the sequence revealed two possible AUG initiation codons (nucleotide position 1 and nucleotide position 10) within the open reading frame, both of which have weak

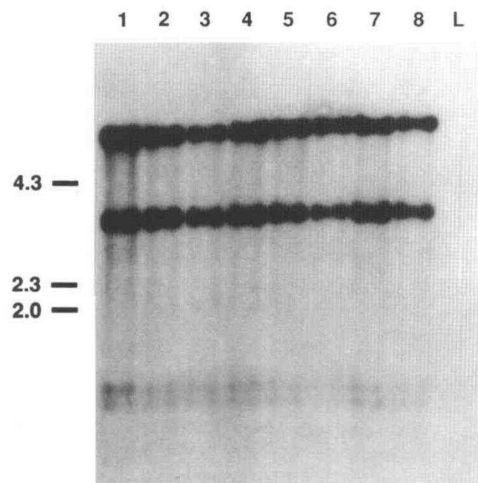

Figure 2. DNA Gel Blot Hybridization Analysis Showing Cosegregation of the T-DNA Insert with the *ga4* Mutation.

DNA was isolated from leaf tissue of F_3 progeny of individual F_2 (*ga4-2* × *tt2*) plants exhibiting the semidwarf phenotype (lanes 1 to 8). The DNA was digested with HindIII, separated by electrophoresis, bound to nylon filters, and then hybridized to ^{32}P-labeled pBIN19 plasmid that contains the T-DNA border sequences. The hybridization pattern correlates with the T-DNA insert and the T-DNA–plant DNA junctions. Four fragments associated with the T-DNA insert are visible in all plants (lanes 1 to 8) and cosegregated with the semidwarf phenotype. Molecular length markers are given at left in kilobases. L, canonical, wild type, Landsberg *er.*

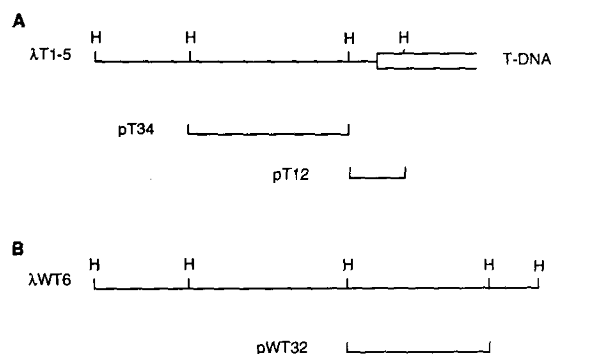

Figure 3. Restriction Maps of the Genomic Clones and Subclones Used To Isolate the *GA4* Gene.

(A) Genomic clone λT1-5. This clone was derived by screening the *ga4-2* genomic library using the ^{32}P-labeled pBIN19 vector as a probe. The 1.2-kb HindIII fragment subclone pT12 contains the T-DNA–plant DNA junction and was used to identify the insertion site by sequencing into the T-DNA insertion break point. The plant sequences from the flanking 3.4-kb HindIII fragment subclone pT34 were used to isolate the corresponding wild-type genomic clone λWT6.
(B) Genomic clone λWT6. The subclone pWT32, which corresponds to the the T-DNA insertion site in λT1-5, was used as a probe to screen the wild-type leaf cDNA library and the *ga4-1* genomic library. All constructs were subcloned into pBluescript KS−.
H, HindIII restriction site.

homology with the "Kozak" consensus sequence for translation initiation (Kozak, 1987; Lütcke et al., 1987).

To confirm that the sequence determined is indeed the *GA4* locus, genomic fragments from the other allele, *ga4-1*, were isolated and sequenced. The *ga4-1* allele was generated by EMS mutagenesis in the same genetic background, Landsberg *er*. Sequence analysis of *ga4-1* indicates that the EMS-induced mutation occurs at nucleotide 659 (Figure 4), resulting in a single nucleotide change from G to A and a corresponding amino acid change from cysteine to tyrosine. This nucleotide change in the coding region, which led to the amino acid change, is presumably responsible for the *ga4-1* mutation.

Data base searches indicated sequence similarity with several genes. An alignment of the amino acid sequence of GA4 with barley flavanone-3-hydroxylase (Meldgaard, 1992) showed 24% amino acid identity, and alignment with pumpkin gibberellin 20-oxidase (Lange et al., 1994) showed 22% amino acid identity (Figure 5). In addition, alignment with the amino acid sequence of 1-aminocyclopropane-1-carboxylate oxidase (ethylene-forming enzyme) from avocado (McGarvey et al., 1990) showed 18% amino acid identity (data not shown). The *GA4* locus therefore probably encodes a hydroxylase; this conclusion is in agreement with a biochemical study that indicated *GA4* most likely encodes the protein 3β-hydroxylase in the GA biosynthetic pathway of Arabidopsis (Talon et al., 1990).

The *ga4* Mutant Overexpresses *ga4* mRNA

To study the pattern of *GA4* gene expression, total RNA was isolated from different tissue types, and RNA gel blots were hybridized with a *GA4*-specific probe (Figure 4). A 1.4-kb transcript was detected in roots, flowers, and siliques when 2 μg of total RNA from each sample was loaded on the gel (Figure 6A). The same-size transcript was detected in leaves when

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     TCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCGGATAAGAAAAAAAACACAAAC
     ATCTATCAAATTTACAAAGTTTTAAAACTAATTAAAAAAGAGCAAG

   1 ATGCCTGCTATGTTAACAGATGTGTTTAGAGGCCATCCCATTCACCTCCCACACTCTCAC   60
   1 M  P  A  M  L  T  D  V  F  R  G  H  P  I  H  L  P  H  S  H    20

  61 ATACCTGACTTCACATCTCTCCGGGAGCTCCCGGATTCTTACAAGTGGACCCCTAAAGAC  120
  21 I  P  D  F  T  S  L  R  E  L  P  D  S  Y  K  W  T  P  K  D    40

 121 GATCTCCTCTTCTCCGCTGCTCCTTCTCCTCCGGCCACCGGTGAAAACATCCCTCTCATC  180
  41 D  L  L  F  S  A  A  P  S  P  P  A  T  G  E  N  I  P  L  I    60

 181 GACCTCGACCACCCGGACGCGACTAACCAAATCGGTCATGCATGTAGAACTTGGGGTGCC  240
  61 D  L  D  H  P  D  A  T  N  Q  I  G  H  A  C  R  T  W  G  A    80

 241 TTCCAAATCTCAAACCACGGCGTGCCTTTGGGACTTCTCCAAGACATTGAGTTTCTCACC  300
  81 F  Q  I  S  N  H  G  V  P  L  G  L  L  Q  D  I  E  F  L  T   100

 301 GGTAGTCTCTTCGGGCTACCTGTCCAACGCAAGCTTAAGTCTGCTCGGTCGGAGACAGGT  360
 101 G  S  L  F  G  L  P  V  Q  R  K  L  K  S  A  R  S  E  T  G   120

 361 GTGTCCGGCTACGCGTCGCTCGTATCGCATCTTTCTTCAATAAGCAAATGTGGTCCGAAG  420
 121 V  S  G  Y  A  S  L  V  S  H  L  S  S  I  S  K  C  G  P  K   140

 421 GTTTCACCATCACTGGCTCGCCTCTCAACGATTTCCGTAAACTTTGGCCCCAACATCACC  480
 141 V  S  P  S  L  A  R  L  S  T  I  S  V  N  F  G  P  N  I  T   160
               ▼
 481 TCAACTACTGCGATATCGTATGAAGAGTACGAGGAACATATGAAAAAGTTGGCATCGAAA  540
 161 S  T  T  A  I  S  Y  E  E  Y  E  E  H  M  K  K  L  A  S  K   180

 541 TTGATGTGGTTAGCACTAAATTCACTTGGGGTCAGCGAAGAAGACATTGAATGGGCCAGT  600
 181 L  M  W  L  A  L  N  S  L  G  V  S  E  E  D  I  E  W  A  S   200
                                                               *
 601 CTCAGTTCAGATTTAAACTGGGCCCAAGCTGCTCTCCAGCTAAATCACTACCCGGTTTGT  660
 201 L  S  S  D  L  N  W  A  Q  A  A  L  Q  L  N  H  Y  P  V  C   220

 661 CCTGAACCGGACCGAGCCATGGGTCTAGCAGCTCATACCGACTCCACCCTCCTAACCATT  720
 221 P  E  P  D  R  A  M  G  L  A  A  H  T  D  S  T  L  L  T  I   240

 721 CTGTACCAGAACAATACCGCCGGTCTACAAGTATTTCGCGATGATCTTGGTTGGGTCACC  780
 241 L  Y  Q  N  N  T  A  G  L  Q  V  F  R  D  D  L  G  W  V  T   260

 781 GTGCCACCGTTTCCTGGCTCGCTCGTGGTTAACGTTGGTGACCTCTTCCACATCCTATCC  840
 261 V  P  P  F  P  G  S  L  V  V  N  V  G  D  L  F  H  I  L  S   280

 841 AATGGATTGTTTAAAAGCGTGTTGCACCGCGCTCGGGTTAACCAAACCAGAGCCCGGTTA  900
 281 N  G  L  F  K  S  V  L  H  R  A  R  V  N  Q  T  R  A  R  L   300

 901 TCTGTAGCATTCCTTTGGGGTCCGCAATCTGATATCAAGATATCACCTGTACCGAAGCTG  960
 301 S  V  A  F  L  W  G  P  Q  S  D  I  K  I  S  P  V  P  K  L   320

 961 GTTAGTCCCGTTGAATCGCCTCTATACCAATCGGTGACATGGAAAGAGTATCTTCGAACA 1020
 321 V  S  P  V  E  S  P  L  Y  Q  S  V  T  W  K  E  Y  L  R  T   340

1021 AAAGCAACTCACTTCAACAAAGCTCTTTCAATGATTAGAAATCACAGAGAAGAATGA    1077
 341 K  A  T  H  F  N  K  A  L  S  M  I  R  N  H  R  E  E  *      359

     TTAGATAATAATAGTTGTGATCTACTAGTTAGTTTGATTAATAAATTGTTGTAAATGATT
     TCAGCAATATGATTTGTTTGTCCTCAA
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Figure 4. Nucleotide and Deduced Amino Acid Sequence of the *GA4* cDNA Clone.

The *GA4* cDNA is encoded by 1077 nucleotides and has an open reading frame of 359 amino acids (Genome Sequence Data Base Accession No. L37126). The position of the intron as deduced from a comparison of cDNA and genomic sequences is indicated by ▼. The site of T-DNA insertion is within the intron. The EMS-induced mutation occurs at nucleotide 659 (*) and results in a single nucleotide change from G to A and an amino acid change from cysteine to tyrosine. The underlined area indicates the sequence of the polymerase chain reaction–labeled probe used for RNA gel blot analysis.

4 μg of total RNA was loaded on the gel (Figure 6B). The gene was expressed ubiquitously in the different tissues examined (root, leaf, flower, and silique), but the message was most abundant in the silique.

There was differential expression between the wild type and mutants in 4-week-old rosette leaves. There was three- to fourfold more message expressed in the EMS-induced *ga4-1* plants when compared with the wild type, but no message was detected in the T-DNA–tagged *ga4-2* plants (Figure 6B). The overexpression of *ga4* message, which was detected in the *ga4-1* plants, can be repressed by the application of 10^{-5} M GA_3 on the rosette leaves of Arabidopsis. The transcriptional repression was detected at 8 hr after the initial treatment and lasted for up to 24 hr (Figure 6C).

DISCUSSION

We have isolated the Arabidopsis *GA4* gene by T-DNA tagging. The T-DNA insertion mutant *ga4-2* and the EMS-induced mutant *ga4-1* both contain sequence alterations in the gene. The changes in the mutant alleles interfere with normal transcription. The deduced amino acid sequence of the GA4 protein shows similarity with the sequences of flavanone-3-hydroxylase and 1-aminocyclopropane-1-carboxylate oxidase from a variety of plant species (Deikman and Fischer, 1988; McGarvey et al., 1990; Britsch et al., 1992; Meldgaard, 1992). On the basis of this sequence similarity, we propose that the *GA4* gene encodes a hydroxylase involved in GA biosynthesis. This conclusion agrees with information based on biochemical studies (Talon et al., 1990) that showed that the Arabidopsis *ga4* mutant had reduced levels of the 3-hydroxy and 3,13-hydroxy GAs and that it accumulates the 13-hydroxy GAs and the non-3,13-hydroxy GAs, with some exceptions. Therefore, the *GA4* gene most likely encodes a protein with 3β-hydroxylation activity, but its actual enzymatic activity remains to be demonstrated. Presumably, a similar activity and gene sequence will be found for the cognate genes corresponding to *GA4* in agronomically important crop plants.

The overexpression of *ga4* message in the EMS-induced *ga4-1* mutant and transcriptional regulation by exogenous GA_3 are novel findings with regard to the regulation of the GA biosynthetic pathway. The terminal GAs in Arabidopsis are GA_1 and GA_4, which are effective in causing stem elongation (Talon et al., 1990). GA_3 has been shown to be present at low levels in vegetative tissue of maize. GA_3 is biosynthesized from GA_{20} via GA_5; GA_1 is the product of GA_3 in maize (Fujioka et al., 1990). There is no evidence of GA_3 biosynthesis in Arabidopsis, but experiments show that exogenous GA_3 is active in promoting stem elongation in Arabidopsis and in other species. The biological activity may be induced by either GA_3 itself or the terminal GAs, such as GA_1, as shown in the proposed pathway in maize (Fujioka et al., 1990). In wild-type plants, the concentrations and proportions of the cellular GAs are maintained by the balance between synthesis and utilization. In the *ga4-1* plant, this balance is perturbed by the mutation and the concomitant reduction in the catalytic activity

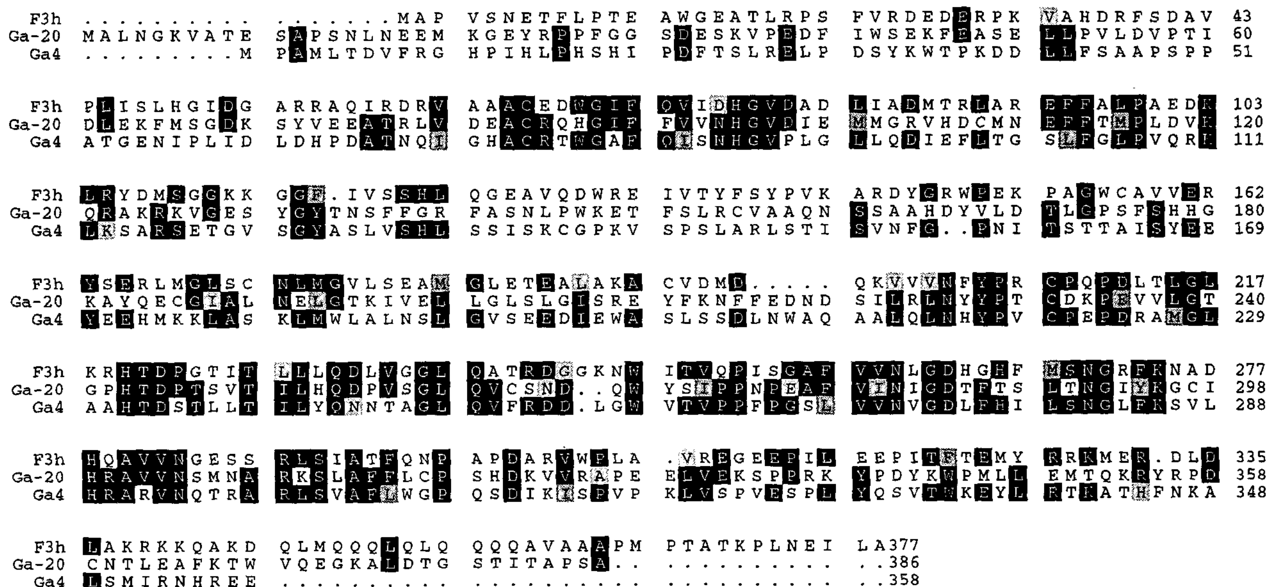

Figure 5. Amino Acid Sequence Comparison of GA4, Barley Flavanone-3-Hydroxylase, and Pumpkin Gibberellin 20-Oxidase.

Alignment is shown for the deduced amino acid sequences of the *GA4* gene (Ga4) from Arabidopsis, flavanone-3-hydroxylase (F3h) from barley (Meldgaard, 1992), and gibberellin 20-oxidase (Ga-20) from pumpkin (Lange et al., 1994). Identical residues are shown in black boxes with white lettering; conserved amino acids are indicated by light shading. Dots were introduced to optimize alignment.

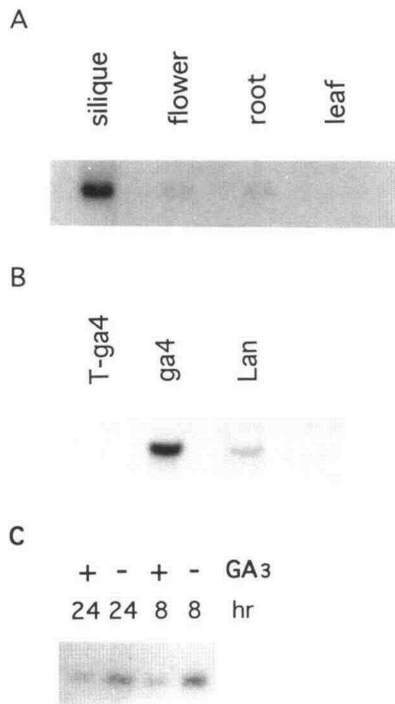

Figure 6. RNA Gel Blot Analysis of *GA4* Gene Expression in Arabidopsis.

RNA blots were subjected to hybridization with a ^{32}P-labeled polymerase chain reaction *GA4*-specific probe (see Figure 4, legend).
(A) *GA4* gene expression in different tissues (silique, flower, root, and leaf). Approximately 2 μg of total RNA from each sample was loaded on the gel.
(B) *ga4* gene expression in 4-week-old rosette leaves of *ga4-2* plants (T-ga4), *ga4-1* plants (ga4), and wild-type Landsberg *er* (Lan). Approximately 4 μg of total RNA from each sample was loaded on the gel.
(C) *ga4* gene expression in *ga4-1* with (+) or without (−) exogenous GA_3. The *ga4-1* plants were sprayed with 10^{-5} M GA_3, and leaf samples were taken 8 and 24 hr after the treatment. Approximately 2 μg of total RNA from each sample was loaded on the gel.

of the 3β-hydroxylase, which leads to the accumulation of GA_9 and GA_{20} and the reduction in GA_4 and GA_1, respectively. The mutated gene would lead either to translation of the mutant form of the protein (presumably inactive or less active) or to no translation at all. The overexpression of *ga4* message, as detected in the *ga4-1* plants and the repression of transcription by exogenous GA_3, indicates a transcriptional feedback regulatory mechanism. One hypothesis to explain these results in the *ga4-1* plants is that the regulatory domain of the GA4 protein is intact but the reduced levels of endogenous GA_4 and GA_1 diminish the feedback control by the terminal GAs. The application of exogenous GA_3, which leads to the accumulation of terminal GAs in Arabidopsis, restores the feedback mechanism.

It has been previously established that 3β-hydroxylation is important in the regulation of stem growth (Phinney and Spray, 1982; Ingram et al., 1984). The results of the studies presented here indicate that, in addition to the critical roles the properties and compartmentalization of the active GAs play in stem growth, molecular regulatory mechanisms also play an important part in the control of GA biosynthesis.

METHODS

Plants and RNA and DNA Isolation

An ethyl methanesulfonate (EMS)-induced *ga4* mutant (*ga4-1*) was obtained from M. Koornneef (Agricultural University, Wageningen, The Netherlands). A T-DNA–tagged *ga4* mutant (*ga4-2*) was generated by *Agrobacterium tumefaciens* root transformation with the pBIN19 vector (Bevan, 1984). Plants were grown under greenhouse conditions using a 16-hr light/8-hr dark cycle. Tissue for DNA and RNA isolation was harvested at ~ 3 to 4 weeks after planting and before bolting, frozen in liquid nitrogen, and stored at −70°C. Genomic DNA was isolated using the methods of Watson and Thompson (1986). Total RNA was isolated using the methods of Ausubel et al. (1989).

Library Construction and Screening

The genomic libraries for the T-DNA insertion mutant (*ga4-2*) and EMS-induced mutant (*ga4-1*) were constructed in λFIX II vectors (Stratagene) and packaged using Gigapack II Gold packaging extracts (Stratagene). The *ga4-1*, *ga4-2*, and Landsberg *erecta* (*er*) genomic libraries and the Landsberg cDNA library were plated on strain ER1458. Plaque lifts were made using Hybond filters (Amersham Corp.), which were then autoclaved for 2 min. Filters were hybridized with probes as described for DNA and RNA gel blot analysis.

DNA Subcloning and Sequencing

Bacteriophage λ DNA was prepared from ER1458 lysates according to the miniprep method of Grossberger (1987). DNA fragments were subcloned into pBluescript KS− vectors (Stratagene) and used to transform JM109.

Double-stranded DNA was isolated from plasmid clones and purified by CsCl banding. Sequencing was performed using α-^{35}S-dATP and Sequenase (United States Biochemical Corp.) according to the manufacturer's protocol for double-stranded DNA sequencing. Sequence analysis was performed using the GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, WI) and the Blast network service of the National Center for Biotechnology Information (Bethesda, MD).

DNA and RNA Gel Blot Analysis

Electrophoresis of DNA was in Tris–acetate–EDTA buffer with subsequent transfer in 25 mM $NaHPO_4$ to Biotrans filters (International Chemical and Nuclear Corp., Aurora, OH). RNA samples were electrophoresed

in agarose gels containing RNase inhibitor using 3-(*N*-morpholino)-propanesulfonic acid–EDTA buffer and transferred to nylon filters as was done for DNA gel blot analysis. Filters were UV cross-linked using a Stratalinker (Stratagene) and baked for 1 hr at 80°C.

Radioactive probes were separated from unincorporated nucleotides using a 1-mL Sephadex G-50 spin column (Pharmacia) and denatured in a microwave oven (Stroop and Schaefer, 1989). Prehybridization for 1 hr and hybridization overnight were performed at 65°C in the hybridization buffer described by Church and Gilbert (1984). Filters were washed once for 15 min in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) at room temperature and then twice for 30 min in 0.1 × SSC, 0.1% SDS at 60°C. The damp filters were autoradiographed at −80°C using intensifying screens. Filters were stripped twice in 2 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2% SDS at 70°C for 30 min prior to reprobing (Church and Gilbert, 1984).

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