Molecular Organization and Tissue-Specific Expression of an Arabidopsis 14-3-3 Gene

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The 14-3-3 proteins, originally described as mammalian brain proteins, are ubiquitous in eukaryotes. We isolated an Arabidopsis 14-3-3 gene, designated GRF1-GF14x (for general regulatory factor1-G-box factor 14-3-3 homolog isoform chi), and characterized its expression within plant tissues. Sequence comparison of the GRF1-GF14x genomic clone with other 14-3-3 proteins demonstrated that the extreme conservation of 14-3-3 residues in several domains is encoded by the first three exons. The highly variable C-terminal domain is encoded by a divergent fourth exon that is unique among 14-3-3 homologs, suggesting that exon shuffling might confer gene-specific functions among the isoforms. The anatomical distribution and developmental expression of the Arabidopsis 14-3-3 protein were examined in transgenic plants carrying a GRF1-GF14x promoter-β-glucuronidase construct. GF14x promoter activity was observed in the roots of both seedlings and mature plants. In immature flowers, GF14x promoter activity was localized to the buds. However, as the flowers matured, GF14x promoter activity was restricted to the stigma, anthers, and pollen. In immature siliques, GF14x promoter activity was initially localized to styles and abscission zones but was subsequently observed throughout mature siliques. In situ hybridization demonstrated that GF14x mRNA expression was prominent in epidermal tissue of roots, petals, and sepals of flower buds, papillae cells of flowers, siliques, and endosperm of immature seeds. Thus, plant 14-3-3 gene expression exhibits cell- and tissue-specific localization rivaling that observed for 14-3-3 proteins within the mammalian brain.

INTRODUCTION

The 14-3-3 proteins are acidic dimeric proteins that constitute nearly 1% of the total soluble protein found in the brain (Moore and Perez, 1967). In addition to their abundance in mammalian brains, 14-3-3 proteins have been reported in a number of tissues in a variety of organisms (Aitken, 1995; Ferl, 1996).

An assortment of activities and functions has been attributed to the 14-3-3 proteins. In some of the early work describing the 14-3-3 proteins, researchers found that they functioned as kinase-dependent activators for tyrosine and tryptophan hydroxylases and were involved in the biosynthesis pathways for catecholamine and serotonin (Ichimura et al., 1988; Isobe et al., 1991). The 14-3-3 proteins have also been described as potential components of signal transduction pathways involving calcium, calcium-dependent protein kinases, and other secondary messengers (Ichimura et al., 1991; Isobe et al., 1992; Roth et al., 1994). Several 14-3-3 proteins have been associated with cell cycle control and mitogenic signaling pathways involving Raf-1 and Bcr (Fantl et al., 1994; Fu et al., 1994; Reuther et al., 1994; Shimizu et al., 1994). In plants, 14-3-3 proteins are associated with transcription activators in the nucleus, suggesting a possible role in transcriptional regulation (de Vetten et al., 1992; Lu et al., 1992). In addition, a 14-3-3 isoform from Arabidopsis has been shown to activate protein kinase C (PKC) and tryptophan hydroxylase and to bind calcium (Lu et al., 1994a, 1994b). All of these various functions are united by the theme of protein–protein interactions involving kinases, suggesting conserved modes of fundamental 14-3-3 activities among organisms.

However, the specificity of different activities associated with the 14-3-3 proteins may be dependent on, or reflected in, cellular localization. One of the outstanding hallmarks of members of the mammalian 14-3-3 gene family is the high degree of specific expression in select cell types. Because the individual function(s) of the different homologs is not known, the first step toward elucidating the role of the specific 14-3-3 proteins is to look for expression patterns unique to each homolog. Early studies reported that the 14-3-3 ζ isoform was localized to the Purkinje cells of the rat cerebellum (Watanabe et al., 1991). Subsequent studies using in situ hybridization determined that the 14-3-3 β and γ isoforms were differentially localized to select tissues within the rat. The γ isoform was more brain specific, whereas the β isoform was localized to lymphoid-specific tissues (Watanabe et al., 1993).
In addition to cell specificity, animal 14-3-3 isoforms have been shown to be regulated during developmental phases of select organs. Developmental biologists working with 14-3-3 proteins in rat pineal glands found that expression of the 14-3-3 ε isoform was greatest at birth but decreased steadily through-out development. However, the expression levels of other 14-3-3 isoforms increased as the pineal glands matured (Roseboom et al., 1994). In another example, the distribution patterns of the 14-3-3 δ and ζ isoforms were distinctly different in the developing rat brain. Before birth, both the δ and ζ 14-3-3 isoforms were expressed at high levels in the brain, whereas 21 days after birth only the ζ isoform was expressed at high levels (Watanabe et al., 1994). These studies indicate that select members of the 14-3-3 family are likely to be developmentally regulated in addition to having tissue/cell specificity.

To approach an understanding of the role that 14-3-3 proteins have in plants, we isolated a genomic clone encoding a member of the 14-3-3 family in Arabidopsis. The anatomical distribution and developmental profile of the 14-3-3 homolog GF14x (G-box factor 14-3-3 homolog isoform chi) were examined in transgenic plants carrying the promoter of the genomic clone fused to β-glucuronidase (GUS). Distribution of GF14x mRNA within different Arabidopsis cell types was examined by in situ hybridization with an isoform-specific probe. Our results suggest that whereas 14-3-3 proteins in general are present in most tissues, differential localization of the GF14x isoform demonstrates developmental and tissue-specific expression that may reflect a unique role for GF14x.

RESULTS

Nomenclature of Arabidopsis 14-3-3 Proteins

We have recently proposed nomenclature for the genes encoding the 14-3-3 homologs in Arabidopsis (Rooney and Ferl, 1995; Ferl, 1996). The designation for the genomic sequence consists of the three-letter symbol GRF (general regulatory factor), with the number one designating the first genomic sequence. The cDNA in this study, GF14x, is encoded by the GRF1 gene. To minimize confusion, the full gene reference is GRF1-GF14x.

GRF1-GF14x Gene Sequence and Structure

The complete nucleotide sequence of GRF1-GF14x is shown in Figure 1. The gene is composed of 3250 bp, including 985 bp upstream and 495 bp downstream of the coding region. GRF1-GF14x contains an open reading frame of 801 bp interrupted by three introns (Rooney and Ferl, 1995). The derived amino acid sequence of the GF14x cDNA is composed of 267 amino acids and encodes a protein calculated to be 30 kD. The transcription initiation site was localized by using 5’ rapid amplification of cDNA ends–polymerase chain reaction to identify the 5’ guanine cap.

Within GF14x is a domain resembling a pseudosubstrate for PKC (Figure 1), which originally was thought to inhibit PKC activity (Aitken et al., 1992). However, recent studies involving site-directed mutagenesis of this region did not alter the inhibitory effect (Jones et al., 1995). Also located within GF14x is a highly conserved domain found in plant, animal, and yeast 14-3-3 proteins (Figure 1). Reports of annexin similarities (Aitken et al., 1995), calcium binding–dependent exocytosis (Roth et al., 1994), and binding to PKC (Xiao et al., 1995) have all been implicated with this domain. A domain that has been shown to be involved in calcium binding and that contains a putative EF-hand motif is indicated by the boldface italicized amino acids (Figure 1; Lu et al., 1994c).

A comparison of 19 plant, yeast, and animal 14-3-3 homologs was conducted to assess regions of conservation among the 14-3-3 proteins and the relationship of conservation versus exon location. The nineteen 14-3-3 homologs compared were Arabidopsis GF14x, GF14q, and GF14a (Lu et al., 1994b), Arabidopsis GF14c (K. Wu and R.J. Ferl, unpublished data), maize GF14-12 (de Vetten et al., 1992), rice (Kidou et al., 1993), barley (Brandt et al., 1992), Oenothera PHP-O (Hirsch et al., 1992), tobacco (Chen et al., 1994), yeast brain modulosignal homologs BMH1 and BMH2 (van Heusden et al., 1995), Drosophila (Swanson and Ganguly, 1992), sheep kinase C inhibitor protein KCIP (Aitken et al., 1990), bovine 14-3-3β (Isobe et al., 1991), rat 14-3-3ζ, sheep 14-3-3ε (Roseboom et al., 1994), human 14-3-3 T-cell (Nielsen, 1991), human 14-3-3ζ (Zupan et al., 1992), and rat 14-3-3ε (Martin et al., 1994).

Figure 2 provides this comparison as a graph of conserved amino acids at each residue within this selected group of 14-3-3 proteins. Above the graph is a diagram showing the exon arrangement of the GRF1-GF14x gene and the α-helical designations that were obtained from the crystal structure of the human 14-3-3ζ isoform (Liu et al., 1995). Exons 1 and 3 contain the annexin-like domain, the pseudosubstrate domain, and other sequences that are highly conserved among the different 14-3-3 proteins, as well as regions (such as the extreme N terminus) that are variable. Exons 2 and 3 exhibit an extraordinarily high degree of overall conservation with large blocks of amino acids that are 100% identical among the 19 proteins. Included in these exons are the domains most likely involved in contacts with phosphorylated proteins, such as Raf kinase and tryptophan hydroxylase (Ichimura et al., 1995; Luo et al., 1995). The last exon, exon 4, exhibits far less conservation than the first three exons, with <30% identity.

Expression of GF14x in Various Organs and Tissues of Arabidopsis

The genomic sequence of GRF1-GF14x was used to provide information on the anatomical distribution of 14-3-3 proteins in plants. Developmental regulation of GF14x was evaluated by using the full-length promoter of GRF1-GF14x fused to the GUS reporter gene. Specificity of cell-type expression of mRNA GF14x was evaluated by in situ hybridization, using an isoform-specific probe.
Cloning and Expression of a 14-3-3 Gene

Figure 1. Genomic Sequence and Organization of Arabidopsis GRF7-GF14x.

The complete DNA sequence of the GRF7-GF14x gene, including introns and 5' and 3' flanking regions, is shown. The nucleotide at position +1 corresponds to the first nucleotide of the cDNA. The nucleotides upstream from the +1 position are indicated by negative numbers. The derived amino acid sequence is shown in the single-letter code below the DNA sequence. The boxed residues encode a potential pseudosubstrate for PKC. Underlined residues identify the annexin-like domain. The translation stop codon is indicated by an asterisk. The EF-hand-like motif is indicated by uppercase boldface italic letters. The promoter fragment that was fused to the GUS reporter gene for developmental studies contains residues from position -856 to +60. The gene-specific probe that was used for in situ hybridization studies was constructed from position +166 to +397. The GenBank accession number for the genomic sequence is U09377, and the updated sequence of the GF14x cDNA is accession number L09112.
Figure 2. Comparison of 19 Plant, Yeast, and Animal 14-3-3 Homologs to Assess Regions of Conservation Relative to Exon Structure.

The amino acid residues of the GF14x protein are plotted against the amount of conservation that each residue shares with 18 14-3-3 proteins. The amino acids of GF14x are given on the abscissa, and the number of isoforms that agree at each position is plotted on the ordinate. Above the graph is a diagram showing the exon arrangement of the GRF1-GF14x gene and the nine designated α-helical regions of the human 14-3-3 ζ protein, according to Liu et al. (1995).

Transgenic Plants

A 916-bp fragment of the Arabidopsis GRF1-GF14x promoter was fused to the GUS reporter gene to determine the anatomical distribution and developmental regulation of GF14x (Figure 1, residues −856 to +60). GRF1-GF14x promoter-driven GUS activity in transgenic Arabidopsis organs was localized to roots, flowers, siliques, and imbibed seeds but not to leaves or cotyledons (Figures 3A to 3I). In both young seedlings and mature plants, GRF1-GF14x promoter-driven GUS activity was present throughout the entire root system but did not extend into the hypocotyl or leaf tissue (Figures 3A and 3B). GF14x promoter activity was also noted in the root hairs of mature roots (Figure 3C).

In mature plants, GRF1-GF14x promoter-driven GUS activity was found in the reproductive structures of Arabidopsis but not in the stems or leaves. Within clusters of flowers, prominent GF14x promoter activity was observed in all flower buds (Figure 3D). GRF1-GF14 promoter-driven GUS activity was also observed in the flower bud trichomes. A reduction of GF14x promoter activity was observed in the pedicels in comparison with the flower buds.

Examination of mature Arabidopsis flowers revealed an organ-specific pattern of GRF1-GF14x promoter-driven GUS activity. Figure 3E shows GF14x promoter activity in both the stigma and style of the mature flower. No activity was noted in the petals or sepals of the mature flower. In mature stamens, GF14x promoter activity was localized mainly to the anthers, with some activity observed in the filaments. The mature anthers also contained intensely stained pollen grains within the pollen sac. Pollen grains that were present on papillae cells of flowers from GRF1-GF14x transformants also expressed activity.

GRF1-GF14x expression was shown to be developmentally regulated in Arabidopsis siliques. As the gynoecium matured and the style became morphologically distinct from ovary, GF14x promoter activity remained localized to the style and did not penetrate into the ovary (Figure 3F). Within the style, activity was observed in the stigmatic tissues and papillae cells. The abscission zone in the immature siliques was also an area of intense GF14x promoter activity, which was initiated in the abscission zone and radiated outward (Figure 3G).

Within mature siliques, GF14x promoter activity was detected throughout the entire organ, with intense activity still prominent in the abscission zone and style (Figure 3H). GRF1-GF14x expression was observed in seeds from transformed plants (Figure 3I). Seeds that had been imbibed for 24 hr had activity localized to the area in which the testa had ruptured.

In Situ Hybridization Histochemistry

Based on the organ specificity information from the transgenic Arabidopsis plants, roots, flowers, and siliques were examined for labeled GF14x mRNA accumulation by using in situ
Examination of transformed Arabidopsis revealed an organ-specific pattern of GRF1-GF14\(\chi\) promoter–driven GUS activity. (A) and (B) Histochemical localization of GUS is detectable in the roots of seedlings (A) and mature plants (B). (C) GF14\(\chi\) promoter activity is also detectable in the root hairs. (D) In immature flowers, GF14\(\chi\) promoter activity can be seen throughout the bud. (E) As the flower matures, GF14\(\chi\) promoter activity is present in the anthers and stigmatic tissues. (F) and (G) In immature siliques, GF14\(\chi\) promoter activity is localized to the differentiated style (F) and the abscission zone (G). (H) In fully mature siliques, intense GF14\(\chi\) promoter activity is seen throughout the siliques. (I) GRF1-GF14\(\chi\) promoter–driven GUS activity can also be observed in areas exposed by the ruptured testa of imbibed seeds.
Figure 4. In Situ Expression of GF14α mRNA in Arabidopsis.

Organs from Arabidopsis cv Columbia were sectioned and hybridized with sense or antisense mRNA probes of GF14α.

(A) Flower buds were examined with the sense probe GF14α as control for nonspecific binding.

(B) GF14α mRNA is localized primarily to the sepals and petals of flower buds.

(C) In mature Arabidopsis flowers, GF14α mRNA is localized in the stigmatic papillae, with the greatest concentration of message at the stigmatic surface.

(D) Expression of GF14α message in mature siliques is prominent in the epidermis and developing seed.

(E) In roots, the GF14α message is localized to epidermal tissue.

e, embryo; en, endosperm; ep, epidermis; ov, ovule; p, petals; pc, papillae cells; po, pollen; s, sepals; st, stigmatic tissue; vt, vascular tissue.
An example of the background signal levels in a young flower bud examined with the sense probe GF14x is shown in Figure 4A. GF14x mRNA signals, detected with the antisense probe, were reproducibly greater than the signals observed in sections with sense GF14x probe controls. Dark-field microscopy revealed solid white areas to be tissues that naturally scatter light and do not represent GF14x message.

Longitudinal sections of Arabidopsis flower buds revealed GF14x message throughout tissues (Figure 4B). Concentrated levels of expression were observed in the unopened petals and sepals. GF14x message was also detected in the developing anthers and pistil. Message levels within immature pollen grains were difficult to detect by using in situ hybridization.

When mature Arabidopsis flowers were examined for GF14x expression, message was concentrated in the upper parts of the gynoecium, specifically the stigmatic tissues (Figure 4C). Most of the GF14x mRNA was localized in the stigmatic papillae, with the greatest concentration of message at the stigmatic surface. GF14x expression was also prominent in the short style, in comparison with very low levels of expression observed in the immature ovules.

Examination of mature siliques showed GF14x mRNA expression to be primarily in epidermal tissue of the siliques (Figure 4D). Developing seeds, which were in the torpedo to upturned-U stages, were also expressing GF14x message. Low-level GF14x mRNA expression occurred throughout the immature seed, but high-level expression was prominent in the endosperm.

In Arabidopsis roots, the greatest concentration of GF14x message was localized in the outer cell layers, predominantly epidermal tissue (Figure 4E). In the cortex layers of the root, the level of GF14x mRNA expression was lower in comparison with the epidermal tissues. GF14x message (if present) was impossible to detect in the vascular tissue due to the light-scattering properties of this tissue.

**DISCUSSION**

**Gene Structure**

The 14-3-3 family of proteins contains numerous regions that are highly conserved among plant, animal, human, yeast, and invertebrate families. Comparison of the regions of conservation with the exon structure of Arabidopsis GRF7-GF14x revealed that all of the conserved sequences reside entirely within the first three exons. The first exon of GRF1-GF14x encodes several preserved domains composed of five complete helices. These conserved sites include the annexin-like domain, which may be involved in calcium binding–dependent exocytosis (Morgan and Burgyne, 1992; Aitken et al., 1995), and a region thought to be capable of binding the 14-3-3 protein to PKC (Xiao et al., 1995). The first exon is also the site at which dimerization interface occurs (Lu et al., 1994c; Liu et al., 1995; Xiao et al., 1995; K. Wu and R.J. Ferl, unpublished data).

Even though no specific functional domains have been identified in exon 2, this is the exon that contains the potential phosphorylation site(s) that may interact with Raf-1 kinase (Liu et al., 1995; Xiao et al., 1995). Exon 3 contains the EF-hand–like motif that is highly conserved in all 14-3-3 proteins. The Arabidopsis 14-3-3 homologs alone show a 95% identity at the amino acid level within this exon. The similarity within the first three exons suggests that these domains are required for fundamental mechanistic functions inherent to all 14-3-3 proteins, including interaction with other proteins.

The C-terminal domain, encoded by the fourth exon, is unique among 14-3-3 homologs, suggesting that this region confers the gene-specific functions of the isoforms. We speculate that this region may function in differentiating the activities of not only the Arabidopsis 14-3-3 isoforms but all 14-3-3 proteins and that this specificity may arise by shuffling the fourth exon.

The diversity of the promoters from the different GRF genes may play a critical role in sorting the differential activities of the 14-3-3 proteins. Comparison of GRF1-GF14x promoter to the GRF1, GRF2, and GRF3 promoters of maize (de Vetten and Ferl, 1994) and Arabidopsis (Rooney and Ferl, 1995) did not reveal any significant regions of homology. Based in part on the uniqueness of the GRF promoters, a portion of the GRF1-GF14x promoter was used to identify the anatomical distribution of a GF14x to determine whether cell-specific expression is a characteristic shared by plant and animal 14-3-3 proteins.

**Anatomical Distribution**

In the reproductive organs of Arabidopsis, expression of GRF1-GF14x was localized to select tissues during development. Arabidopsis is a self-fertile genus in which self-incompatibility is not known to occur, so the role(s) of stigmatic tissue and papillae cells in the early events of pollination is not completely understood (Thorsness et al., 1993; Hulskamp et al., 1995). Self-incompatible species, such as Brassica, utilize a specific pollen–papillar cell signaling system that requires biochemically active papillar cells (Kandasamy et al., 1993). The Brassica species self-incompatibility system is based on the activity of a transmembrane receptor protein kinase (Goring and Rothstein, 1992; Stein and Nasrallah, 1993). However, in Arabidopsis the ability of the stigmatic tissues to accept pollen is not based on the phosphorylation state and appears to be developmentally regulated by changes in stigma receptivity (Kandasamy et al., 1993; Preuss et al., 1993).

Expression of GF14x in the reproductive organs raised the question of whether GF14x was expressed under developmental control or in response to reproductive pollination signals. In transformed plants, GRF1-GF14x promoter-driven GUS activity was detected before and after fertilization in the stigmatic tissues and pollen grains. In addition, the male-sterile mutant pistillata was used to evaluate GF14x mRNA expression in the absence of stigma–pollen interaction. GF14x mRNA expression in stigmas of the pistillata mutant was similar to the pattern observed in wild-type Arabidopsis stigmas (data not shown).
presumably indicating a developmental pattern of expression rather than a specific pollen–papillar cell signaling system.

Based on the observations from the in situ hybridization studies, the majority of GF14x message had accumulated in the epidermis or outermost cell layers of expressing organs. Because the epidermis is the first tissue exposed to bacteria, insects, and pathogenic fungi, expression of epidermal-specific genes might be expected. Several 14-3-3 proteins have been found in plants that were challenged with a fungal pathogen or fungal metabolite (Brandt et al., 1992; Korthout and de Boer, 1994). Potential fusococcin binding proteins isolated from several plant species were shown to be 14-3-3 homologs (Aducci et al., 1995). However, there is no direct correlation between localization and function of the 14-3-3 proteins in any system.

The data presented in this report demonstrate a specific anatomical and developmental distribution of a 14-3-3 protein in plants. Arabidopsis transformed with the GRF1-GF14x promoter-driven GUS construct allowed us to study both tissue distribution and developmental regulation. The correlation with in situ hybridization strengthens the assertion that the promoter contains the elements necessary for tissue- and organ-specific expression. Results from this study support the idea that the various 14-3-3 isoforms have separate and distinctive tissue distribution, suggesting that plant 14-3-3 isoforms may indeed have specific roles within individual tissues. These findings indicate that cell-specific expression is a consistent characteristic of 14-3-3 genes in all higher eukaryotes.

METHODS

Isolation of Genomic Clone

An Arabidopsis thaliana genomic library (Clontech, Palo Alto, CA) was plated on Escherichia coli K803 and screened by hybridization at high stringency with 32P-labeled PGF14x (Lu et al., 1994b). The hybridization was performed at 65°C for 18 hr in 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS, and 1% BSA. The filters were washed for 1 hr with three changes of 40 mM sodium phosphate, pH 7.2, 24 mM NaCl, 0.1% SDS, and 1 mM EDTA at 65°C and exposed to Kodak XAR-5 film with an intensifying screen. Hybridizing recombinant phages were rescreened at lower plaque density to plaque purity. Phage particles were isolated, and DNA subsequently was purified.

Subcloning and Sequencing

Relevant DNA fragments were cloned into pGEM-7Z(+)- vectors and subjected to double-stranded nested deletions (Pharmacia, Piscataway, NJ). The 5' end of GRF1-GF14x mRNA was identified by the 5' guanine cap (Bahringer et al., 1994), using 5' rapid amplification of cDNA ends–polymerase chain reaction (Jain et al., 1992). The nucleotide sequence of both strands of the plasmid DNA was determined by automated dideoxy chain termination on an ABI 373 (Applied Biosystems, Foster City, CA), according to the manufacturer's protocols. Nucleotide sequence analysis was performed using SeqEd (Applied Biosystems). Nucleotide and amino acid sequence alignments were performed using GeneWorks (IntelliGenetics, Mountain View, CA).

β-Glucuronidase Reporter Constructs

The promoter from the GRF1-GF14x gene was amplified from a cloned 2.0-kb XbaI HindIII fragment. The amplified 916-bp GRF1-GF14x promoter was subcloned into digested pBl101 (Clontech), which contained the promoterless β-glucuronidase (GUS) gene. The GRF1-GF14x-GUS construct was transferred into Agrobacterium tumefaciens EHA-105 by using a direct transformation protocol (An et al., 1988).

Transgenic Plants

Arabidopsis ecotype RLD plants transformed with GRF1-GF14x promoter–GUS reporter gene were obtained by using a root transformation method (Valvekens et al., 1988). Briefly, sterilized seeds were grown in liquid germination media (GM) for 3 to 4 weeks in the dark at 23°C. Roots were separated from the rest of the plant and placed on callus induction media (CIM) for 5 days. The Agrobacterium strain carrying the GRF1-GF14x promoter–GUS fusion were grown for 3 days at 28°C. Roots were removed from the CIM plates, cut into 1-cm sections, soaked in the Agrobacterium strain for 3 min, blotted on filter paper, and placed on shoot induction media (SIM) with sucrose. Shoots were co-cultivated on SIM under continuous light at 23°C. After 3 days, roots were rinsed in liquid CIM for 3 min and placed on SIM with sucrose. Green calli appeared within 1 to 2 weeks, and shoots appeared within 3 weeks. Shoots were removed and placed on root induction media for 3 days. Shoots were then placed on GM plates containing Phytagel (Sigma) until roots formed; then plantlets were transferred to soil. F2 or F3 progeny were used for GUS assays.

Histochemical GUS Assays

Histochemical assays of GUS activity were done essentially as described by Stomp (1992), with minor modifications. Briefly, tissue samples were incubated 1 hr to overnight with 5-bromo-4-chloro-3-indolylβ-D-glucuronide cyclohexylammonium salt (X-gluc; Gold Biotechnology, Inc., St. Louis, MO). Samples were cleared and fixed in 70% ethanol. Cleared plant material was photographed using dark-field microscopy.

In Situ Hybridization

Flowers, immature siliques, leaves, roots, and stems of Arabidopsis cv Columbia or the Arabidopsis pistillata mutant (Arabidopsis Biological Resource Center, Columbus, OH) were fixed for 24 to 48 hr in FAA (10% formaldehyde-fresh [Sigma], 5% acetic acid, and 50% ethanol). Sections were transferred through a dehydration series containing tertiary-butyl alcohol and ethanol. Tissue samples were embedded with Para-Plast Plus (Sigma), sectioned to 10 μm on a Leitz (Wetzlar, Germany) microtome, and mounted on Probe-On Plus slides (Sigma). A 243-bp EcoRI-HindIII fragment from the GF14x cDNA was subcloned from pUC18 into pGEM3Z (Promega). Sense and antisense RNA probes for in situ hybridization were produced by using an SP6/T7 transcription kit (Boehringer Mannheim) and labeled with 35S-UTP (Du Pont–New England Nuclear). In situ hybridizations were performed essentially according to the methods of Cox and Goldberg (1988). Slides were prehybridized to reduce nonspecific binding, immersed upright in the hybridization mix, and incubated at 55°C for 18 hr. Slides were then washed at 65°C to reduce background and exposed to NTB-2 photographic emulsion (Kodak) for 4 days at 4°C. Slides were subse-
quently developed and stained with toluidine blue. Slides were examined using a Nikon (Melville, NY) optiphot light microscope. Photomicrographs were taken with Fujicolor (Tokyo, Japan) Super G color film, using dark-field microscopy.

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