Tagging and Cloning of a Petunia Flower Color Gene with the Maize Transposable Element Activator

George Chuck, Tim Robbins, Charanjit Nijjar, Ed Ralston, Neal Courtney-Gutteson, and Hugo K. Dooner
DNA Plant Technology Corporation, 6701 San Pablo Avenue, Oakland, California 94608

We report here the use of the maize transposable element Activator (Ac) to isolate a dicot gene. Ac was introduced into petunia, where it transposed into Ph6, one of several genes that modify anthocyanin pigmentation in flowers by affecting the pH of the corolla. Like other Ac-mutable alleles, the new mutation is unstable and reverts to a functional form in somatic and germinal tissues. The mutant gene was cloned using Ac as a probe, demonstrating the feasibility of heterologous transposon tagging in higher plants. Confirmation that the cloned DNA fragment corresponded to the mutated gene was obtained from an analysis of revertants. In every case examined, reversion to the wild-type phenotype was correlated with restoration of a wild-type-sized DNA fragment. New transposed Acs were detected in many of the revertants. As in maize, the frequency of somatic and germinal excision of Ac from the mutable allele appears to be dependent on genetic background.

RESULTS

Isolation of a Variegated Flower Color Mutation in a Petunia Line Carrying Ac

Several independent transformants carrying Ac have been generated in V26, a highly inbred, purple-flowered genetic line that was obtained from the collection at the Free University of Amsterdam (Robbins et al., 1991). The binary vector used in transformation, pJJ4411, has been described previously.
(Keller et al., 1993a). In addition to a hygromycin resistance transformation marker, this vector contains the streptomycin phosphotransferase (SPT)::Ac excision marker between the right and left T-DNA borders. The maize element Ac interrupts the SPT gene and prevents its expression (Jones et al., 1989). In several plants, such as tobacco and Arabidopsis, this marker is a useful visual indicator of somatic and germinal Ac activity (Jones et al., 1989; Dean et al., 1992; Keller et al., 1992). In petunia, however, the streptomycin germination screen is not as reliable. It can be used to enrich for plants carrying transposed Ac elements (trAc), but such plants need to be confirmed by DNA gel blot analysis. This procedure results in a greater than 10-fold enrichment for trAc in petunia (T. Robbins and N. Courtney-Gutterson, unpublished results).

One of the plants analyzed (3057.12) carried two Ac elements in heterozygous condition: one still in its resident site in a T-DNA (Jones et al., 1989) and the other at an unlinked chromosomal location, into which it had integrated following a secondary transposition event from a different T-DNA. Plant 3057.12 was selfed to make homozygous the transposed Ac (trAc) element and, therefore, any mutation caused by the trAc insertion. When the self-progeny were planted, a new, variegated flower color phenotype was found to segregate as a simple recessive Mendelian trait. As can be seen in Figure 1A, the variegated flower phenotype is striking; darkly colored (revertant) sectors, outlined by white rims, stand out sharply against the pale colored (mutant) background of the corolla. The color of the background and the revertant sectors varies depending on the residual genotype. In segregants from outcrosses to other genetic lines, the background color was blue and the revertant color red; however, in all flowers, a white rim separates the revertant sector from the mutant background. Borders of different colors are rarely seen in examples of anthocyanin variegation. When they are seen, the rims tend to be more, not less, pigmented than the areas they delimit. The formation of new anthocyanin pigments in the border cells has been attributed to the diffusion of accumulated intermediates from adjacent cells (McClintock, 1951; Rhoades, 1952). The presence of white rims in the variegated flowers also suggests that compounds can diffuse into the border cells from adjacent cells, i.e., the effect of the mutated gene on anthocyanin pigmentation is not strictly cell autonomous.

**Evidence That the New Mutation Is Tagged by Ac**

The following evidence indicated that the new variegated mutation had arisen as a consequence of an Ac transposition event, and was, therefore, tagged by Ac.

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**Figure 1. Flower Phenotypes.**

(A) Variegated petunia corolla produced by a plant carrying the new unstable allele.
(B) Plant with a variegated mutant flower (bottom) and solidly colored revertant flower (top).
(C) Branch showing an older variegated flower (left) with a faded background color relative to that of the recently opened variegated flower (right).
Cosegregation with Ac-Hybridizing Band

The mutation cosegregated with a new Ac-hybridizing band in DNA gel blots of the self-progeny of plant 3057.12. DNA from the variegated (mutant) and solidly colored (parental) progeny was analyzed by digestion with different enzymes and hybridization with an Ac probe. In an EcoRI digest, a new Ac-hybridizing band was found that cosegregated with the new variegated phenotype. As seen in the genomic DNA gel blot shown in Figure 2A, a 4.7-kb band is present in every variegated plant (V1 to V6) but only in some solidly colored siblings (S1 to S3).

The larger (~5-kb) band represents the second unlinked Ac element. This band can be seen to segregate in both the variegated and the solidly colored siblings. Among those variegated plants that received both Ac fragments, the intensity of the 4.7-kb band relative to the 5-kb band is either the same (V5) or double (V2 to V4), suggesting that the new, 4.7-kb band is homozygous but that the 5-kb band can be either homozygous (V5) or heterozygous (V2 to V4). Conversely, some solidly colored progeny (S3 and S7) appear to be homozygous for the 5-kb but not the 4.7-kb band.

In all, 26 variegated siblings were analyzed and all showed the new, Ac-hybridizing band at approximately the same relative intensity. Of 25 solidly colored siblings analyzed, 16 had the 4.7-kb band, a result in agreement with the proportion of heterozygotes for the new mutation (two-thirds) expected within the solidly colored class. These data indicate that the new mutation is linked to the trAc band (χ² = 10.2, P < 0.01). Although no recombinants were found in the self-progeny, the resolution of this type of F₂ linkage data (repulsion phase, complete dominance) is limited, so the 95% confidence interval for the estimate of ρ, the recombination fraction, is large (ρ = 0; CI = 0–0.34).

Homozgyosity for the Ac-Tagged Fragment

All of the mutant plants were homozygous for the Ac-tagged DNA fragment. The Ac-homologous, 4.7-kb EcoRI fragment, containing part of Ac and DNA adjacent to the Ac insertion, was cloned into the vector pZapII (Stratagene). A restriction map of this fragment (and of the adjacent 6.8-kb EcoRI fragment subsequently isolated) is shown in Figure 2C. The BstXI-EcoRI fragment flanking Ac in the 4.7-kb Ac fragment was labeled and used to reprobe the blot shown in Figure 2A. If the variegated plants are indeed homozygous for the new, 4.7-kb Ac band, they should lack the allelic wild-type fragment, which, conversely, should be present in all of the solidly colored siblings. The DNA gel blot presented in Figure 2B confirms this expectation. The solidly colored progeny now show a 7-kb band of identical mobility to the band seen in the wild-type V26 inbred parent. In contrast, the variegated siblings either lack that band or show it at a very reduced intensity. The weak band present in the variegated progeny can be attributed to occasional somatic excisions of Ac.
As anticipated, those solidly colored progeny that did not show a 4.7-kb Ac-homologous band in Figure 2A are homozygous for the V26 wild-type fragment. Solidly colored progeny that did show a 4.7-kb Ac-homologous band are heterozygous for the V26 fragment. The overall segregation obtained from having scored the progeny of plant 3057.12 with the two different probes (Ac and its flanking sequence) is as follows. Among the 25 solidly colored progeny analyzed, 16 were Ac/+; 9 were Ac/Ac. Among the 26 variegated progeny, all were Ac/Ac. This more complete genotypic classification of the F2 progeny significantly reduces the size of the 95% confidence interval for \( p = 0; \) CI = 0–0.05) and demonstrates that the new mutation is, in fact, closely linked to Ac.

**Reversion to Wild-Type Phenotype**

Reversion of the mutation to the wild-type phenotype was correlated with restoration of a wild-type–sized DNA fragment. Confirmation that a mutation is in fact tagged by Ac can be sought from an analysis of revertants because an excision of Ac that restores the wild-type phenotype should also produce a DNA fragment of the original wild-type size. Progeny from variegated plants were grown and screened for somatic and germinal reversion events. Branches with solidly colored flowers, representing large somatic revertant sectors, were occasionally seen on plants producing mostly variegated flowers (Figure 1B). DNA from the solidly colored and variegated branches of one such plant was prepared and analyzed by DNA gel blotting. The blot shown in Figure 3A was probed with the BstXI-EcoRI fragment flanking Ac (Figure 2C).

Two bands of roughly equal intensity can be seen in the lanes containing DNA from a flower and a leaf that were borne on a revertant branch (lanes 4 and 5). One is a 7-kb, wild-type–sized band and the other, a 4.7-kb band, which also hybridizes to Ac (data not shown). This observation indicates that the revertant sectors are heterozygous for the original Ac-induced mutation and a revertant allele produced by excision of Ac during development of the chimeric plant. Capsules borne on the revertant branches produced solidly colored and variegated individuals in a 3:1 ratio, confirming that the reversion event was heritable. The lanes containing DNA from a flower and leaf that were borne on a variegated branch (Figure 3A, lanes 2 and 3) show, in contrast, a strong 4.7-kb Ac band and a faint 7-kb band. The latter band probably represents empty sites generated by somatic excisions of Ac during the formation of the variegated flower. Capsules borne on variegated branches produced, as expected, mostly variegated progeny.

Plants with only solidly colored flowers have been obtained among the progeny of variegated plants at frequencies ranging from 6 to 27%, indicating that the new mutation is also germinally unstable and reverts frequently to the wild-type state. Representative reversion data are shown in Table 1. To date, 10 independent germinal revertants have been analyzed by DNA gel blots, confirming the observations made earlier for the somatic revertant sectors. Figure 3B illustrates the analysis of three such germinal revertants. All the revertants (lanes 2, 5, and 6) showed the 7-kb, wild-type–sized band in addition to the 4.7-kb band. Therefore, they are heterozygous for a revertant allele and the original Ac-induced mutation. The segregating variegated siblings (Figure 3B, lanes 3 and 4), on the other hand, showed only the 4.7-kb band; they are homozygous for the Ac mutation, as expected from their phenotype. In addition, six of eight revertants analyzed had new Ac bands, indicating that the excised Ac elements continue to be capable of reinsertion (data not shown).

We concluded from the above evidence that the new variegated petunia mutant arose from the transposition of the maize element Ac into a gene affecting flower color.

### Table 1. Frequency of Germinal Revertants

<table>
<thead>
<tr>
<th>Family</th>
<th>Pedigree</th>
<th>Number Variegated Plants</th>
<th>Number Solidly Colored Plants</th>
<th>Frequency Germinal Revertants</th>
</tr>
</thead>
<tbody>
<tr>
<td>3426</td>
<td>V26 x M59</td>
<td>73</td>
<td>9</td>
<td>0.11</td>
</tr>
<tr>
<td>3428</td>
<td>V26 x M59</td>
<td>103</td>
<td>25</td>
<td>0.19</td>
</tr>
<tr>
<td>3434</td>
<td>V26 x M59</td>
<td>35</td>
<td>13</td>
<td>0.27</td>
</tr>
<tr>
<td>3466</td>
<td>V26 x M59</td>
<td>51</td>
<td>9</td>
<td>0.15</td>
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<tr>
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<td>V26</td>
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<td>2</td>
<td>0.08</td>
</tr>
<tr>
<td>3468</td>
<td>V26</td>
<td>91</td>
<td>6</td>
<td>0.06</td>
</tr>
</tbody>
</table>

\( ^{\text{a}} \text{Number of solidly colored plants to total number of plants.} \)

**Figure 3. DNA Gel Blot Analysis of Revertants.**

Genomic DNA (6 μg) was digested with EcoRI, separated by electrophoresis on 1% agarose gels, transferred to nylon membranes, and probed with the BstXI-EcoRI fragment from DNA flanking the Ac insertion (bar in Figure 2C). Molecular length markers are given at left in kilobases. *(A)* Somatic sector shown in Figure 1B. Lane 1, 3057.12 parent plant; lanes 2 and 3, leaf and flower, respectively, borne on a variegated branch; lanes 4 and 5, leaf and flower, respectively, borne on a solidly colored branch.

*(B)* Germinal revertants. Lane 1, V26 inbred line; lanes 2, 5, and 6, solidly colored progeny of a variegated plant; lanes 3 and 4, variegated progeny of same plant.
The Gene Tagged by Ac Affects the Acidity of the Corolla

Several considerations suggested that the new Ac-tagged mutant was a ph-type mutation, i.e., a mutation in one of the several genes known to affect anthocyanin pigmentation in petunia by changing the acidity of the corolla (de Vlaming et al., 1983; Wiering and de Vlaming, 1984). First, in certain genetic backgrounds, the revertant sectors in the variegated flowers appear red, whereas the mutant background has a distinct bluish hue. This color change is reminiscent of that brought about by the ph mutations in petunia. These mutations cause a bluing of the corolla by increasing the vacuolar pH in the anthocyanin-accumulating cells (Wiering and de Vlaming, 1984). Anthocyanins in solution undergo a similar shift from red to blue as the acidity decreases.

Second, the new mutation affects the pH of the corolla in a manner similar to the known ph mutant ph1. This was established by comparing the corolla pH of mutant and revertant plants that arose in the self-progeny of a variegated plant, which was also Hf1 Ph1/hf1 ph1. Hf1 and Ph1 are closely linked genes on chromosome 1 that affect pigmentation of the corolla. Hf1 controls hydroxylation at the 5' position of the anthocyanin B ring and causes a bluing of the corolla. Ph1 increases acidity in the vacuoles of the corolla and produces a more reddish hue. The Hf1 Ph1/hf1 ph1 heterozygote was obtained from an outcross of a variegated V26 plant (Hf1 Ph1) to line M59 (hf1 ph1). Among Hf1 segregants, the pH of the corolla was higher in the mutant plants (5.89 ± 0.02) than in the revertant plants (5.57 ± 0.03), a result that suggested that the new mutation altered the acidity of the corolla. This increase in pH can be compared to that caused by the ph1 mutation in the same segregating family. Because ph1 and hf1 are only 1 centimorgan apart, hf1 segregants should also be ph1/ph1 and, therefore, were used to compare the effects on corolla pH of ph1 and of the new mutation. Two revertant progeny of the hf1 class (and, presumably, ph1/ph1) were recovered; both had high corolla pH values (5.90 ± 0.05), similar to those measured in the variegated flowers of the Hf1 class.

Third, in some variegated plants the color of the flower fades with aging (Figure 1C), a phenomenon that has also been observed in petunia lines carrying certain ph mutations in conjunction with the Fa allele (Wiering and de Vlaming, 1984). The pigments present in the faded corollas of the variegated line were compared with those present in the faded corollas of a ph4 mutant line. In both cases, the faded flowers accumulated a phenolic compound fluorescing blue under UV light. This compound was absent in extracts of recently opened, nonfaded flowers.

Fourth, the new mutation has a pleiotropic effect on seed development, an effect that has also been associated with some ph mutations (Wiering and de Vlaming, 1984). When examined under 30x magnification, seeds borne on mutant plants appear abnormal. Some are shriveled or irregularly shaped and the vast majority are variegated. The seed coat of normal petunia seeds is uniformly pigmented and reticulated. As shown in Figure 4, the mutant seed coat is largely unpigmented and lacks the honeycomb network of normal seeds, except for areas of varying size where the pigmented and reticulated peripheral structure is restored. The variegated seed phenotype of the Ac-induced mutation can be readily explained as another manifestation of somatic instability: the normal sectors on the mutant seed coats would form as a result of Ac excisions that occurred during seed development.

The observations given above cumulatively suggested that Ac had become inserted in one of the Ph genes. We conducted allelism tests with existing ph mutants and established that the variegated mutant was capable of complementing all of the mutants tested, except for ph6, as illustrated in Figure 5. Therefore, we have designated the new, Ac-tagged ph6 mutation ph6-m1(Ac). The petunia line carrying the standard ph6 mutation used in the complementation test is W160. The flower phenotype conditioned by the ph6-m1(Ac) allele in outcrosses to W160 is clearly different from that produced in the pure V26 line or in a mixed V26/M59 genetic background. Only small sectors can be seen (Figure 5) due to reversion events that occur late in flower development. The seed phenotype is similarly affected: a few of the seeds borne on the outcross plants show traces of pigmentation, but the majority are unpigmented (data not shown).

The ph6-m1(Ac) Mutation Encodes an Altered Form of a Flower-Specific Transcript

To detect a Ph6 transcript, total RNA was prepared from Ph6 and ph6-m1(Ac) flower buds and leaves. The RNAs were separated on a 1.1% agarose gel, blotted onto a nylon membrane,
Figure 5. Flower Phenotypes from the Allelism Test to ph6.

(A) Ph6/ph6 (top) and ph6-m1(Ac)/ph6 (bottom), both in a V26/W160 genetic background.

(B) Magnification of bottom flower in (A) showing the small revertant sectors on a mutant background.

Figure 6. RNA Gel Blot of Wild-Type and Mutant Flower Buds and Leaves.

Total RNA (10 μg) was separated on a 1.1% agarose gel, transferred to a nylon membrane, and hybridized sequentially to three probes.

(A) Hybridization to the EcoRI-BamHI DNA fragment on the left side of the Ac insertion (Figure 2C).

(B) Hybridization to the flower-specific CHS-A probe (Koes et al., 1989).

(C) Hybridization to a wheat rDNA probe.

Lane 1, mutant leaf; lane 2, 2-cm mutant flower bud; lane 3, wild-type leaf; lane 4, 2-cm wild-type flower bud. Top arrow indicates the position of 28S rRNA; bottom arrow, position of 18S rRNA.

and probed with the EcoRI-BamHI fragment that extends from the BamHI site in Ac to the left of the insertion site (Figure 2C). The corresponding RNA gel blot is shown in Figure 6A. An ~2.8-kb transcript is detected in wild-type flower buds (lane 4). In the mutant flower bud (lane 2), only a trace of the 2.8-kb transcript can be seen; the major signal is given, instead, by a less than 2-kb transcript. Possibly, alternate splicing caused by the Ac insertion accounts for the multiple transcripts seen in the mutant (Wessler, 1988). No transcript can be detected in either mutant or wild-type leaves (lanes 1 and 3), suggesting that the Ph6 gene is expressed preferentially in flowers.

The RNA gel blot was rehybridized with the petunia flower-specific chalcone synthase probe CHS-A (Koes et al., 1989) after washing away the first probe (Figure 6B). As expected, transcripts of the same size and intensity were detected in mutant and wild-type flower buds (lanes 2 and 4, respectively) but not in mutant and wild-type leaves (lanes 1 and 3, respectively). This result shows that the mutant RNA sample was not degraded and confirms the flower-specific nature of the Ph6 transcript. Finally, Figure 6C shows the comparable 28S rRNA signal given by the four RNA samples when the RNA gel blot was rehybridized with a wheat rDNA probe, confirming that the four lanes were loaded with approximately the same amount of RNA.
DISCUSSION

The new petunia unstable allele described here represents a validation of the heterologous transposon tagging strategy. The ph6-m7(Ac) mutation arose in a random or nondirected Ac-tagging experiment in which a petunia plant carrying a trAc in heterozygous condition was selfed to reveal the mutation. We do not know the linkage relationship between the Ph6 gene and the donor locus from which Ac transposed because the trAc element was not initially inserted in a readily scored excision marker. The random transposon tagging approach resembles T-DNA tagging (Feldman, 1991) in that new, recessive mutations at nontargeted loci are revealed after the insertion is made homozygous. Given Ac’s propensity to transpose to linked sites in most systems (Greenblatt, 1984; Dooner and Belachew, 1989; Jones et al., 1990; Dooner et al., 1991b; Keller et al., 1993b), a directed tagging approach using an Ac element linked in cis to the target locus is preferable in experiments that aim to tag specific genes. However, the process of identifying a close linkage between an introduced T-DNA and the gene of interest is time consuming, so the first few mutations tagged with heterologous transposons in dicots can be expected to arise from random tagging experiments such as this one.

The frequency of revertant sectors in the variegated flowers and of germinal revertants among the progeny of the mutant indicate that Ac excision from the ph6-m7(Ac) allele is not a rare event. Ac appears to excise as frequently from the petunia Ph6 gene as it does from maize genes, such as Bronze and Waxy. In addition, Ac transposition, i.e., excision and reinsertion, was demonstrated in six of eight germinal revertants. This ratio of recovery of trAc elements relative to excisions is also similar to what has been reported in maize (McClellan, 1956; Greenblatt, 1984; Dooner and Belachew, 1989) and other dicots (Jones et al., 1990; Dooner et al., 1991b; Dean et al., 1992; Keller et al., 1992).

The residual genetic background has been shown to affect both somatic and germinal excision of Ac in maize (Brink and Nilan, 1952) and may also do so in petunia. The developmental timing of Ac excision from ph6-m7(Ac), as evidenced by the size of the revertant sectors, is considerably delayed in a V26/W160 background relative to a V26 or a V26/M59 background (Figure 5). Similarly, the frequency of germinal reversion appears to be higher in a V26/M59 hybrid background than in a V26 inbred background (Table 1). The reduced variegation observed in the V26 × W160 outcross plants may also be partly due to lower Ac dosage because these plants are heterozygous, rather than homozygous, for the ph6-m7(Ac) allele. A positive dosage effect of Ac on somatic variegation has been previously documented for the engineered SPT:Ac gene in both tobacco and Arabidopsis (Jones et al., 1989; Dean et al., 1992; Keller et al., 1992).

The Ph6 gene tagged by Ac modifies anthocyanin pigmentation in the corolla. A Ph6-specific transcript was detected in flowers, but not leaves, indicating that the gene is preferentially expressed in corolla cells. However, because the ph6 mutation also affects the phenotype of the seed coat (Figure 4), the Ph6 gene must also be expressed during seed development. At this time, we do not know the gene's primary function. We have isolated several clones from a petunia flower bud cDNA library and are in the process of sequencing them. Comparison with other gene sequences may provide a clue to the function of Ph6.

The white rims around the revertant sectors in the variegated flowers remain a puzzle. Certain forms of anthocyanins have been shown to appear red at low pH, colorless at higher pH values, and blue at pH values near neutrality (Timberlake and Bridle, 1975). If the white rim is due to an intermediate pH value at the border between mutant and revertant tissue, we thought it would be possible to recreate this condition in vitro by preparing anthocyanin extracts of the variegated flowers and then examining color over a pH range from 2 to 6. When this was done, the expected shift from red to blue was observed as the pH was increased; however, no pH was found that gave a colorless solution. It appears that the particular anthocyanin form found in these extracts of petunia flowers is sufficiently stable not to become colorless at any acidic pH value. We are left to speculate that a diffusible compound essential for the stability of anthocyanins is deficient in mutant tissues, possibly as a consequence of a defective transport system. Normal functioning cells at the borders of the revertant sectors would deplete that compound from the adjacent mutant cells, thus preventing the accumulation of anthocyanins in the latter. In agreement with this model, we have observed that the white rim's border is sharp on the side of the revertant sector but diffuse on the side of the mutant background. This is what would be expected if the cells in the white rim were genotypically mutant and were depleted of the pigment-stabilizing compound by the adjacent normal cells.

METHODS

Constructs

The binary vector plasmid pJJ4411 is described in detail elsewhere (Keller et al., 1993a).

Plant Genotypes

V26 petunia plants were transformed with *Agrobacterium tumefaciens*LBA4404 carrying the binary vector plasmid 4411. Other petunia lines used in this work were M59 and W160, all obtained from the collection maintained at the VUA (Free University, Amsterdam).

Nucleic Acid Extraction and Analysis

DNA was extracted from 4- to 6-cm flower buds by a CTAB procedure (Dooner et al., 1991b). Approximately 6 μg of DNA was digested with the appropriate restriction enzymes, separated on 1% agarose gels,
and blotted to Duralon-UV membranes (Stratagene). The DNA gel blots were hybridized to random primer-labeled probes. RNA was extracted from ground tissue resuspended in 5 M guanidinium thiocyanate (Schmidt et al., 1981). Ten micrograms of total RNA was separated on 1.1% agarose gels and blotted to Duralon membranes. The RNA gel blots were hybridized to random primer-labeled probes.

Genomic Cloning

DNA was extracted from pooled leaf nuclei of variegated plants V1 and V6 (Figure 2). Approximately 40 μg of DNA was digested with EcoRI and size fractionated by centrifugation through a 20 to 40% glycerol gradient. Fractions containing the 5-kb- and 6-kb-hybridizing bands and size fractionated by centrifugation through a 20 to 40% glycerol were hybridized to random primer-labeled probes. RNA was extracted from ground tissue resuspended in 5 M guanidinium thiocyanate (Schmidt et al., 1981). Ten micrograms of total RNA was separated on 1.1% agarose gels and blotted to Duralon membranes. The DNA gel blots were hybridized to random primer-labeled probes.

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