A Dissociation Insertion Causes a Semidominant Mutation That Increases Expression of TINY, an Arabidopsis Gene Related to APETAL2

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A novel transposon-tagging strategy designed to recover dominant gain-of-function alleles was performed with Arabidopsis by using a Dissociation element with a cauliflower mosaic virus 35S promoter transcribing outward over one terminus. Lines containing transposed copies of this transposon were screened for mutants, and a semidominant mutation affecting plant height, hypocotyl elongation, and fertility was recovered. The pleiotropic effects of this mutation appear to result from a general reduction in cell expansion, and some of the effects are similar to those caused by supplying exogenous ethylene or cytokinin to wild-type seedlings. In addition, the arrangement of cells in some organs, such as the etiolated hypocotyl, is disorganized. The mutation was called tiny, and the affected gene was cloned by first using transposon sequences to isolate the mutant allele. The predicted protein product of the TINY gene shows strong homology with the DNA binding domain of a recently identified class of plant transcription factors. This domain, called the APETALA2 domain, was initially identified as a duplicated region within the APETAL2 gene of Arabidopsis and then as a conserved region between APETAL2 and the ethylene responsive element binding proteins of tobacco. In the mutant allele, the Dissociation element is inserted in the untranslated leader of the TINY gene, 35 bp from the ATG, and the mutant contains a novel transcript that initiates from the cauliflower mosaic virus 35S promoter within the transposon. This transcript is present in greater abundance than the wild-type TINY transcript; therefore, the semidominant tiny mutation most likely results from increased, or ectopic, expression of the gene.

INTRODUCTION

Dominant mutations causing increased or ectopic transcription can provide novel mutant phenotypes by affecting genes in which recessive loss-of-function mutations have no obvious effect. They also can increase our understanding of genes identified by loss-of-function alleles by indicating whether expression of a single gene is sufficient to direct a particular process. In plants, this is exemplified by the ovulata mutation of Antirrhinum, which alters the identity of sepals and petals by causing ectopic expression of the PLENA gene in the first two whorls of the flower (Bradley et al., 1993). Another example is the knotted1 (kn1) mutation of maize, which was initially defined by several gain-of-function mutations that result in the formation of undifferentiated cells on the leaves. This phenotype results from the presence of the KN transcript in leaf veins of mutant but not wild-type plants (Smith et al., 1992). Alleles of both kn1 and ovulata are caused by insertion of transposable elements into introns of the respective genes, suggesting that the insertions prevent sequences in the introns acting to reduce transcription of the genes in certain cell types (Hake, 1992; Bradley et al., 1993).

We performed a mutant screen specifically designed to isolate dominant mutations by making use of a modified Dissociation (Ds) transposon. This transposon carries a cauliflower mosaic virus (CaMV) 35S promoter transcribing out of the element over one terminus. Insertion of the element near the 5' end of a gene can result in expression from the CaMV 35S promoter rather than from the gene's own transcriptional signals. This system is analogous to that found in retroviruses and related retrotransposons. A strong promoter at the end of these elements can cause mutations by altering the expression of adjacent host genes (e.g., Payne et al., 1982). In plants, the maize transposable element Mutator also was shown to contain a promoter that can cause the expression of adjacent genes. Transcription of the Hcf106 gene from this promoter was shown to result in a phenotype similar to that of the wild type rather than a novel phenotype (Barkan and Martienssen, 1991). However, a modified T-DNA vector carrying several copies of an enhancer derived from the CaMV 35S promoter adjacent to the T-DNA border can produce gain-of-function mutations causing novel phenotypes (Hayashi et al., 1992). The enhancers cause adjacent genes to be overexpressed, and this system was used successfully to screen for mutations that enabled transformed cells to grow independent of auxin in...
tissue culture and then to isolate the affected genes (Walden et al., 1994).

The maize transposable elements Activator/Ds (Ac/Ds) were used previously in transgenic plants to isolate recessive loss-of-function mutations in Arabidopsis (Bancroft et al., 1993; Long et al., 1993a; James et al., 1995), petunia (Chuck et al., 1993), tobacco (Whitham et al., 1994), and tomato (Jones et al., 1994) and to isolate genes based on their patterns of expression (Smith and Fedoroff, 1995; Springer et al., 1995). In this study, we describe the semidominant tiny mutation that is caused by insertion of the Ds element carrying the CaMV 35S promoter and consequent overexpression of an adjacent Arabidopsis gene. The mutation affects cell shape and cell expansion. The effects on hypocotyl elongation and leaf epidermal cell shape are similar to those caused by the constitutive triple response 1 mutation (Kieber et al., 1993), which disrupts the negative regulation of ethylene responses. We show that the TINY protein is related to a class of plant transcription factors that were previously shown to be involved in Arabidopsis flower development and the regulation of gene expression in response to ethylene in tobacco.

RESULTS

Construction of a Ds Element Designed to Cause Dominant Gain-of-Function Mutations

A Ds element designed to increase the transcription of adjacent genes was constructed by inserting the CaMV 35S promoter 246 bp from one end of the transposon (Figure 1; see Methods). The promoter was inserted so that transcription would occur out of the element and no ATG codons would be present between the transcription start site of the CaMV 35S promoter and the end of Ds. The translation of transcripts initiated from this promoter therefore should not be initiated prematurely within transposon sequences. In addition, the Ds carries a hygromycin resistance gene, so its inheritance can be easily followed phenotypically. The transposon was named Ds(HYG 35S) to indicate that it contains both the hygromycin resistance gene and the CaMV 35S promoter. The transposon was inserted in both orientations within the untranslated leader of a streptomycin phosphotransferase gene (SPT) that confers resistance to the antibiotic streptomycin (Figure 1). In one orientation (oriA), the CaMV 35S promoter transcribes toward the SPT gene; this construct was designed to determine whether the promoter is able to cause the expression of the adjacent resistance gene. In the other orientation (oriB), the SPT gene should be inactivated by insertion of the transposon; this can therefore be used as an assay for Ds excision (Jones et al., 1989). Both Ds::SPT constructions were assembled in a binary vector containing a neomycin phosphotransferase II gene (NPTII) that confers kanamycin resistance on plant cells.

Both constructions were introduced into Arabidopsis plants by selection for kanamycin resistance (see Methods). A total of eight independent transfectants were made containing the transposon in the orientation (oriA) such that the promoter transcribes toward the SPT gene, whereas an additional five transformants were made containing the transposon in oriB. Seed derived from these transformants were sown on agar plates containing kanamycin, hygromycin, or streptomycin, and the ratio of sensitive-to-resistant seedlings was scored in each case. These ratios are shown in Table 1. All transformants containing the transposon in oriA produced streptomycin-resistant progeny, whereas all of the progeny containing the transposon in the reverse orientation were streptomycin sensitive. Previous experiments showed that Ac and Ds elements lacking the CaMV 35S promoter and inserted at the same position in both orientations inactivated the SPT gene (e.g., Jones et al., 1991; Swinburne et al., 1992). Therefore, the data presented here demonstrate that the CaMV 35S promoter within the Ds element is capable of activating the SPT gene and presumably other genes when the transposon is inserted in an appropriate position and in the correct orientation.

Identification of a Semidominant Dwarfing Mutation in a Population Carrying a Transposed Ds Element

Ds(HYG 35S) oriB transformants (Table 1) that contained a single copy of the Ds T-DNA at one locus were identified by gel

![Figure 1. Structure of the Ds(HYG 35S) T-DNAs.](image)

(A) The T-DNA carrying Ds(HYG 35S) in oriA. The CaMV 35S promoter within the transposon transcribes toward the SPT gene.

(B) The T-DNA carrying Ds(HYG 35S) in oriB. The CaMV 35S promoter within the transposon transcribes away from the SPT gene. The NPTII, HPT, and SPT genes that encode resistance to kanamycin, hygromycin, and streptomycin, respectively, are shown. The promoter of the nopaline synthase (nos) gene drives transcription of the HPT gene and is denoted by an open box and an arrow pointing toward HPT. Ds is shown as a triangle with an arrow denoting each terminus. The CaMV 35S promoter within Ds is shown below an arrow that indicates the direction of transcription. The other CaMV 35S promoter enables expression of the SPT gene after Ds(HYG 35S) excision.
Table 1. Proportion of Antibiotic-Resistant Seedlings among the Progeny of Transformants Carrying Ds(HYG 35S) in OriA or OriB

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Kanamycin</th>
<th>Hygromycin</th>
<th>Streptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of</td>
<td>Ratio</td>
<td>No. of</td>
</tr>
<tr>
<td></td>
<td>Seedlings</td>
<td></td>
<td>Seedlings</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriA-1</td>
<td>70:0</td>
<td>100% res</td>
<td>ND⁹</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriA-2</td>
<td>41:18</td>
<td>2.3:1</td>
<td>79:23</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriA-3</td>
<td>152:0</td>
<td>100% res</td>
<td>110:0</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriA-4</td>
<td>99:29</td>
<td>3.4:1</td>
<td>ND</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriA-5</td>
<td>710:123</td>
<td>5.8:1</td>
<td>ND</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriA-6</td>
<td>52:14</td>
<td>3.7:1</td>
<td>50:17</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriA-7</td>
<td>232:57</td>
<td>4.1:1</td>
<td>45:28</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriA-8</td>
<td>142:37</td>
<td>3.8:1</td>
<td>ND</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriB-1</td>
<td>94:25</td>
<td>3.8:1</td>
<td>58:23</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriB-2</td>
<td>78:24</td>
<td>3.3:1</td>
<td>ND</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriB-3</td>
<td>144:64</td>
<td>2.3:1</td>
<td>62:19</td>
</tr>
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<td>Ds(HYG 35S) oriB-4</td>
<td>91:38</td>
<td>2.5:1</td>
<td>ND</td>
</tr>
<tr>
<td>Ds(HYG 35S) oriB-5</td>
<td>137:49</td>
<td>2.8:1</td>
<td>ND</td>
</tr>
</tbody>
</table>

⁹ Resistant (res) to sensitive (sens).
⁸ ND, not determined.

To confirm the prediction that these three classes corresponded to plants homozygous, heterozygous, or lacking the mutation, the genotypes of 35 wild-type plants and 35 plants of intermediate height were tested by self-fertilizing the plants and scoring their progeny. As expected, if the genotypes were as predicted, all 35 wild-type plants produced only wild-type progeny, whereas all of the intermediate plants produced progeny of all three classes. The mutation present in these lines was named tiny, and plants homozygous or heterozygous for the mutation are shown in Figure 2 and are compared with the wild type.

Description of the tiny Mutant Phenotype

To quantify the difference in height between wild-type plants and those homozygous or heterozygous for tiny, 527 plants from a population made by self-fertilizing a heterozygote were grown, and the distance between the last rosette leaf and the top of the main inflorescence was measured. A similar experiment was performed with a control population of wild-type plants, and their heights were compared with those of the segregating population. The progeny of the tiny heterozygote fell into three classes, corresponding to the three phenotypes described above, and the tallest of these three groups showed the same distribution of sizes as the wild-type population (Figure 3A).

Wild-type seedlings germinated in the dark show an etiolation response, including an elongated hypocotyl formed by increased cell expansion (Reed et al., 1993). To study whether the tiny mutation prevents this elongation, a segregating population was germinated in the dark and their hypocotyls were measured. As shown in Figure 3B, three phenotypes, represented by short, medium, and long hypocotyls, were detected in this population. This analysis suggested that tiny prevents the etiolation response in the hypocotyl. By using scanning electron microscopy, hypocotyls of mutant seedlings germinated in the light or dark were compared with those of wild-type plants and of elongated hypocotyl (hy3) and...
The Plant Cell

Figure 2. Comparison of tiny Mutants and Wild-Type Plants.
From left are tiny homozygote, tiny heterozygote, and wild-type plants grown under short-day (10-hr photoperiod) conditions. The tiny homozygote is dramatically shorter than the wild-type plant, and the heterozygote has an intermediate phenotype. Under long-day (16-hr photoperiod) conditions, the tiny mutant shows an even more severe phenotype.

deetiolated (detl) mutants. These are shown in Figure 4. When tiny mutants were germinated in the light, their hypocotyl cells were shorter than those of wild-type seedlings. Furthermore, when the mutants were germinated in the dark, the hypocotyl cells did not elongate in the same way as those of wild-type plants. In addition, although the cells in the hypocotyl epidermis of dark-grown wild-type plants are arranged clearly in files running from the base of the hypocotyl toward the cotyledons, those of tiny are disorganized. The hypocotyl of detl mutants also are shorter than those of the wild type both in the light and dark, but the cells of the det mutants are more regular in shape and arrangement than those of tiny (Figure 4).

Scanning electron microscopy also was performed with leaves of light-grown wild-type and mutant plants. The wild-type leaf epidermis contains a cell type with a characteristically irregular shape (Kieber et al., 1993; Telfer and Poethig, 1994); however, in the leaf epidermis of the mutant, this cell morphology is absent and the cells have a more regular, bulbous shape, as shown in Figure 5. In addition, the cross-sections of leaves, shown in Figure 6, illustrate that the leaves of mutant plants are thicker than those of the wild type.

The flowers of tiny homozygotes show an altered morphology. The mature buds of the homozygous mutant are rounder and less elongated than those of the wild type, and not all of them open. The pistil of the mutant is shorter than that of the wild type, but the anther filaments are much shorter; as a result, the anthers are located below the stigma. Usually the anthers do not dehisce, so no pollen is released. Homozygotes for tiny cannot be cross-fertilized with wild-type pollen and therefore are also female sterile. Heterozygotes show reduced fertility and produce less pollen than do wild-type plants.

Figure 3. Comparisons of Lengths of Stems and Hypocotyls of tiny Mutants and Wild-Type Plants.
(A) The lengths of the bolting stems of a population of plants segregating for the tiny mutation are shown. The plants were grown under long-day conditions. The histogram at the top shows the segregating population, which was made by self-fertilizing a plant heterozygous for tiny. The histogram below shows the distribution of sizes in a population of wild-type plants.
(B) The lengths of the hypocotyls of wild-type plants and of a population segregating for the tiny mutation are shown. Seed of each population were placed on agar plates and then kept in the dark, first at 4°C to promote germination and then at 20°C for 3 weeks to allow germination and hypocotyl elongation to occur. The dotted line marks the wild-type data points, and the solid line is the population segregating for tiny.
Figure 4. Scanning Electron Microscopy of the Hypocotyls of Light- and Dark-Grown Seedlings.

(A) to (D) show light-grown seedlings, and (E) to (H) show dark-grown seedlings.
(A) and (E) Wild-type Landsberg erecta.
(B) and (F) hy3 mutants.
(C) and (G) deft mutants.
(D) and (H) tiny mutants.
(I) to (K) tiny seedlings. (I) and (J) show light-grown seedlings; (K) is a seedling grown in the dark.
The bar in (H) = 100 μm for (A) to (H); the bar in (K) = 1 mm for (I) to (K).

Dwarf mutants defective in gibberellic acid biosynthesis can be corrected by repeated application of a solution of 10⁻⁴ M gibberellin (GA₃) (Koornneef and van der Veen, 1980). A population of plants containing individuals homozygous for tiny were therefore repeatedly sprayed with this solution, but these applications had no effect on their phenotype, indicating that tiny does not block gibberellin biosynthesis.

The tiny Mutation Is Caused by Insertion of a Ds(HYG 35S) Element

To determine whether insertion of the Ds(HYG 35S) transposon was responsible for the tiny mutation, we tested for genetic linkage between the transposon and the mutation and for the restoration of a wild-type phenotype upon excision of the element. Genetic linkage between the transposon and the mutation was demonstrated by showing that the hygromycin resistance gene carried by Ds(HYG 35S) cosegregates with the tiny mutation. One hundred and thirty-five plants whose phenotype suggested that they were heterozygous for tiny were identified, and their genotypes were determined by scoring their progeny both for the presence of tiny mutants and for hygromycin-resistant plants. This showed that all 135 plants were heterozygous for both tiny and Ds(HYG 35S), so no recombination was detected between the mutation and the transposon. Therefore, there is a 95% probability that the transposon and the mutation are within 1.1 centimorgans of each other.

In maize and a variety of transgenic plant species, including Arabidopsis (Bancroft and Dean, 1993; Keller et al., 1993), Ac/Ds elements tend to transpose to sites genetically linked to their starting position. To test whether the tiny mutation is genetically linked to the T-DNA from which the Ds(HYG 35S) element originated, the genetic distance between the hygromycin resistance gene carried by the transposon inserted in or close to the TINY gene and the SPT gene that was activated by excision of the transposon was measured. Plants heterozygous for both the SPT gene and tiny were self-fertilized, and the resulting seed were sown on streptomycin-containing
medium. Then, it was determined how many of the resistant seedlings were also resistant to hygromycin. Eight hundred and sixty-six streptomycin-resistant seedlings were tested, and all of them also were resistant to hygromycin. This demonstrated that the transposon inserted in or close to the TINY gene and the T-DNA from which the element transposed to induce the mutation have a 95% probability of being within 0.5 centimorgans of one another.

A characteristic feature of mutations caused by the insertion of Ds is that the presence of Ac transposase should promote excision of the element, resulting in phenotypic reversion of the mutation. To determine whether the tiny mutation is unstable in the presence of transposase, a fusion of the CaMV 35S promoter to the Ac transposase gene (35S:TPase) was introduced into the tiny mutant by crossing. Seventy-two plants heterozygous for both tiny and 35S:TPase were then self-fertilized, and their progeny were grown in families before being scored for the presence of mutant and wild-type plants. Some of these families contained no individuals showing the characteristically severe phenotype associated with tiny homozygotes, and they also contained a higher proportion of wild-type plants than had previously been reported for the progeny of tiny heterozygotes that did not contain 35S:TPase. This reduction in the number of tiny mutants was dependent on the presence of Ac transposase because it never occurred in the progeny of plants not carrying transposase and is consistent with the occurrence of Ds(HYG 35S) excision reducing the transmission of the mutation to the progeny. Phenotypically wild-type plants carrying revertant alleles were identified by selecting for the presence of the SPT gene, which as shown above is tightly linked to tiny in the mutant and therefore genetically marks the mutant or revertant chromosome (see Methods). To analyze the DNA sequence of the revertant alleles, DNA fragments adjacent to both termini of Ds in the tiny mutant were first isolated by inverse polymerase chain reaction (IPCR; see Methods). These fragments were sequenced, and oligonucleotides were designed and then used to amplify a 310-bp fragment from DNA of wild-type plants and similarly sized fragments from two independently isolated revertants. Figure 7 shows a comparison of the wild-type sequence with those of the IPCR products representing DNA adjacent to Ds(HYG 35S). The transposon was flanked by an 8-bp duplication of plant DNA, which is often the case for Ac/DS elements in maize or transgenic plants (Pohlman et al., 1984; Baker et al., 1986). Both revertant alleles retained the 8-bp duplication, but the central two bases were altered from GG to AC (Figure 6A). This analysis shows that excision of the Ds(HYG 35S) element correlated with reversion of the mutation and therefore that the presence of the transposon was the cause of the tiny mutation.

The TINY Protein Shows Homology with a Class of Transcription Factors That Includes APETALA2 and Ethylene Responsive Element Binding Proteins

The IPCR products containing DNA adjacent to Ds(HYG 35S) were used to identify a cDNA for TINY to determine the position of the transposon within the gene and to provide the sequence of the open reading frame (ORF). Both fragments were com-

Figure 5. Scanning Electron Microscopy of the Leaf Epidermis of a Wild-Type Plant and a tiny Mutant.
(A) Leaf epidermis of a wild-type plant.
(B) Leaf epidermis of a tiny mutant.
The bar in (B) = 100 μm.
Analysis of the tiny Mutation of Arabidopsis

The leaf of the tiny mutant is approximately twice as thick as that of the wild-type plant, probably due to the greater diameter of the cells of the tiny mutant. Bar = 100 μm.

The total length of the cDNA is 1040 bp. Analysis of the sequence identified an untranslated leader of 153 bases, an ORF encoding 218 amino acids, and an untranslated tail of 188 bases followed by a polyadenylation signal (AATAAA; Figure 8). Comparison of the cDNA sequence with that of the IPCR products obtained from each side of Ds showed that the transposon is inserted in the untranslated leader 35 bp upstream of the ATG that initiates the ORF. The IPCR product obtained from the 5' end of the transposon also contained the whole ORF and was colinear with the cDNA, indicating that the gene contains no introns.

The translated ORF of the cDNA was used to screen the SwissProt and GenBank data bases by using the BLAST X and FASTA programs (Pearson and Lipman, 1988). This demonstrated that a 57-amino acid region of TINY, between amino acids 35 and 92, has strong homology with proteins from Arabidopsis, tobacco, rice, and lupin (Figure 9). The function of this domain is known for some of these proteins; for example, the ethylene responsive element binding proteins (EREBPs) of tobacco bind to the promoters of genes that respond to the presence of ethylene, and the conserved domain is both required and sufficient for this binding to occur (Ohme-Takagi and Shinshi, 1995). Similarly, TINY shows homology with a duplicated region of the APETALA2 (AP2) protein, termed the AP2 domain, that has been proposed to be involved in...
Figure 8. The Sequence of the TINY Gene.

The DNA sequence of the TINY cDNA is shown. The 8 bp that are duplicated upon insertion of Ds(HYG 35S) are double underlined. The ORF predicted to encode the TINY protein is shown in boldface letters, and the underlined region shows homology with other plant genes, as illustrated in Figure 9. The predicted amino acid sequence is shown below the ORF, and the nonsense codon that terminates the ORF is marked with an asterisk. The nucleotide sequence has been submitted to the EMBL data base and assigned the accession number X94698.

The Semidominant tiny Mutation Is Probably Caused by Increased Abundance of TINY mRNA

The position and orientation of the Ds(HYG 35S) insertion in the TINY gene of Arabidopsis that is of unknown function but show homology with TINY also are shown. The region of TINY that shows homology with EREBP1/2, EREBP3, and EREBP4 is discussed in the text and shown here. In addition, proteins from rice, lupin, and Arabidopsis are also shown. The region of TINY that shows homology with the 57-amino acid region of TINY and a similar domain in the EREBP/AP2 group of proteins strongly suggests that TINY is also a DNA binding protein. In TINY, the amino end of the region is basic and contains a sequence (RKXN-X,1-RKKS) that might function as a bipartite nuclear localization signal (Raikehl, 1992), whereas in the AP2 protein a putative nuclear localization signal was identified adjacent to the C-terminal end of AP2-R1 (Jofuku et al., 1994). Furthermore, the C terminus of TINY (amino acids 140 to 218) contains a strongly acidic region with an overall negative charge of -13, which is analogous to regions that function as activation domains in a number of transcription factors (Mitchell and Tjian, 1989). An acidic region is also present at the N termini of AP2 (Jofuku et al., 1994), EREBP-1, EREBP-2, and EREBP-4 and at the C terminus of EREBP-3 (Ohme-Takagi and Shinshi, 1995). Taken together, these comparisons suggest that TINY probably functions as a transcription factor.
The tiny Mutation Maps to a Location on Chromosome 5

An experiment described earlier showed that the tiny mutation was genetically closely linked to the T-DNA from which the Ds(HYG 35S) element originally transposed into the TINY gene. Their proximity to one another was confirmed by demonstrating that the TINY gene and a fragment of plant DNA adjacent to the T-DNA both hybridize to the same yeast artificial chromosome clones (R. Schmidt and C. Dean, personal communication). The T-DNA was previously shown to be located on chromosome 5 between restriction fragment length polymorphism marker g6843 and microsatellite marker nga76 (J. Goodrich, unpublished data); therefore, the TINY gene also must be located at this interval.

**DISCUSSION**

We used a novel, modified Ds element in transposon-tagging experiments in Arabidopsis and identified a semidominant mutation that we called tiny. The mutation is caused by the insertion of Ds(HYG 35S), because a copy of the transposon in the mutant cosegregates with tiny and excision of the...
transposon correlates with reversion of the mutation. The dominance of tiny is most likely caused by overexpression of the gene product for the following reasons. The transposon is inserted in the untranslated leader of the gene in the orientation such that the CaMV 3SS promoter transcribes toward the ORF. The gene is expressed at low levels in wild-type plants, but mutants contain high levels of a novel TINY transcript that, based on its size and capacity to act as a template for cDNA synthesis primed by a Ds homologous primer, is initiated from the CaMV 3SS promoter within the Ds. Moreover, insertion of the Ds(HYG 3SS) element in the same orientation and in a similar position in the SPT gene allows efficient expression of streptomycin resistance. The intermediate phenotype of the heterozygote is probably caused by a dosage effect, with two copies of tiny in the homozygotes producing more of the transcript and therefore a more severe phenotype. However, it remains unclear whether the tiny phenotype is caused simply by increased abundance of the transcript or by its expression in cells in which TINY is not normally expressed.

The predicted TINY gene product shows a high degree of homology with the DNA binding domain of a class of plant transcription factors (Jofuku et al., 1994; Ecker, 1995; Ohme-Takagi and Shinshi, 1995; Weigel, 1995). Only one of these proteins required for flower and seedling development of Arabidopsis, AP2, previously has been shown to be affected by mutation (Jofuku et al., 1994), although four other members of this class of proteins were shown to bind to the promoters of tobacco genes whose transcription is activated in response to ethylene (Ohme-Takagi and Shinshi, 1995). The presence of the DNA binding domain in TINY makes it highly probable that the protein binds DNA, and the strongly negatively charged region at the C terminus suggests that it acts by activating the transcription of other genes. The identity of these target genes or the processes in which they are normally involved are unknown (see below).

The tiny mutation appears to cause a reduction in size of mutant plants by affecting cell size and shape. This is apparent in the epidermis of light-grown leaves, where most of the cells are much more regular in shape than in the wild type, in the mutant leaf and hypocotyl, where clusters of small cells are formed that are not present in the wild type, and in the much reduced cotyledon response of the hypocotyl of mutant seedlings growing in the dark, in which the cells do not expand to the same extent that wild-type cells do. This also is likely to be the explanation for the reduced lengths of the stem filaments and pistil of the mutant. Therefore, increased or ectopic expression of TINY apparently can inhibit cell expansion and the differentiation of the shape of some cell types.

The observation that some of the homologous proteins are inducible by environmental conditions, one by the heavy metal cadmium and several by the plant hormone ethylene, raises the possibility that transcription of TINY in wild-type plants might be induced by yet unidentified environmental stimuli and that the low abundance of transcript detected in wild-type plants might represent the level of transcription before induction. In addition, some features of the tiny mutant are similar to those caused by mutations, such as constitutive triple response 1, that cause the ethylene response pathway to be activated constitutively (Kieber et al., 1993). These similar features include a dwarf phenotype, a short, thick hypocotyl when grown in the dark, and a more regular shape of the leaf epidermal cells.

If TINY were a transcription factor at the end of the ethylene response pathway, then expression of TINY from the CaMV 3SS promoter might be expected to cause constitutive activation of the ethylene response pathway. A striking feature of mutations that activate the ethylene pathway in the absence of ethylene is that they show a constitutive triple response: 3-day-old dark-grown seedlings have a short, thick hypocotyl, a short root, and an exaggerated hook (Guzmán and Ecker, 1990). The tiny mutation has a similar effect on the hypocotyl and root but does not cause the characteristic exaggerations in the hook. Therefore, either the TINY gene is not involved in ethylene responses or it is required to activate genes involved in only some of the diverse responses associated with the hormone. Alternatively, TINY could activate genes in response to other growth regulators. For example, application of cytokinin to germinating Arabidopsis seedlings results in some phenotypic abnormalities similar to those shown by tiny: the hypocotyls of dark-grown seedlings are short and thick, and the root is short (Chory et al., 1994). At least in part, cytokinin seems to act by stimulating ethylene synthesis but does not cause a triple response when applied at high concentrations (Gary et al., 1995). Additional work, such as the isolation of loss-of-function alleles, is now required to determine more accurately the function of TINY.

The gene activation system described here represents a powerful method to identify dominant gain-of-function mutations that would be difficult to isolate by traditional mutagenesis strategies because they usually produce loss-of-function alleles. In other systems, such alleles have led to the identification of regulatory genes such as Antennapedia (Schneuwly et al., 1987), c-myc (Payne et al., 1982), and int-1 (Nusse et al., 1984). The need for such a system also is emphasized by the demonstration that many genes do not appear to cause a mutant phenotype when inactivated; for example, of 55 novel genes that were identified during the sequencing of yeast chromosome 11 and disrupted by gene replacement, only 17 caused a clear phenotype when inactivated (Oliver et al., 1992). It is likely that for some of these genes, gain-of-function alleles would cause a phenotype suggesting a function for the gene. This is demonstrated clearly for genes in which loss-of-function alleles caused subtle phenotypes, whereas overexpression of the gene has a dramatic effect: for example, phytochrome A mutations have little effect on plants grown under white light, but under similar conditions, transgenic plants carrying CaMV 3SS:Phytochrome A have a dramatically altered morphology (Kay et al., 1989; Whitelam et al., 1993).

The potential effectiveness of screening for tagged gain-of-function alleles was demonstrated recently in tobacco by using a T-DNA vector carrying multiple CaMV 3SS enhancers near one border sequence. This T-DNA was introduced into protoplasts, and the transformants were selected for mutations...
that allowed cell division in the absence of auxins in cell culture (Hayashi et al., 1992). This experiment led to the isolation and sequencing of such genes (Walden et al., 1994). A potential advantage of the transposon system is that the proximity of the transposon and the coding sequence of the gene in the gain-of-function allele should mean that reactivation of the Ds(HYG 35s) element with the Ac transposase leads, at relatively high frequency, to transposition into the mutated gene or to the formation of adjacent deletions that inactivate the gene. PCR could be used to screen for such alleles, and then it could be determined whether these loss-of-function alleles cause a phenotype (Das and Martienssen, 1995).

**METHODS**

**Plasmid Construction and Plant Transformation**

A Dissociation (Ds) element carrying the hygromycin resistance gene and a cauliflower mosaic virus (CaMV) 35S promoter was constructed and introduced in both orientations into a gene encoding streptomycin phosphotransferase (SPT) as follows. An SsiI-Xhol fragment carrying a hygromycin resistance gene expressed from the neopine synthase promoter was inserted into the unique Xhol site of Activator (Ac). The CaMV 35S promoter was inserted on a BamHI-EcoRI fragment into the BglII (present 248 bp from the 5’ end of Ac in this derivative; the 5’ end is defined as the one from which transcription of the transposase gene is initiated in Ac; Kunze et al., 1987) and EcoRI sites of Ac. This resulted in the CaMV 35S promoter transcribing outward through the 5’ end of Ac. The completed transposon was named Ds(HYG 35s). Then this element was inserted in both orientations between the CaMV 35S promoter and the SPT gene of binary vector pCLO111 (Dean et al., 1992). Both constructs were introduced into Arabidopsis thaliana by using the root transformation procedure of Valvekens et al (1988).

**Transposon-Tagging Strategy**

Plants homozygous for a T-DNA carrying a fusion of the CaMV 35S promoter to the Ac transposase gene (35STPase transformant described by Swinburne et al., 1992) were used. These were crossed with plants homozygous for the T-DNA carrying Ds(HYG 35s) in the orientation such that the CaMV 35S promoter did not transcribe toward the SPT gene (oriB; Figure 1). The F1 plants, which were heterozygous for both T-DNAs, were then self-fertilized, and the F2 progeny were harvested. The F2 seed were sown on GM agar (Valvekens et al., 1988) containing streptomycin (200 μg/mL; Sigma) and hygromycin (40 μg/mL; Calbiochem), as described by Long et al. (1993b). F2 seedlings resistant to both antibiotics were transferred to medium without antibiotics and then to soil. The plants were allowed to self-fertilize and their F3 progeny harvested. These were then sown on GM agar plates and on soil to screen for mutants. The tiny mutant was identified in one such F3 family.

**Inverse Polymerase Chain Reaction and Polymerase Chain Reaction**

Inverse polymerase chain reaction (IPCR) was used to isolate the DNA adjacent to Ds in the tiny mutant. The DNA was cleaved with BstYI and then treated as described by Long et al. (1993a). The primers used to amplify the DNA adjacent to the 5’ end of Ds(HYG 35s) were D74 (5’-TGTAGTTTTATCCCCGATGATTCGCA) and B39 (5’-ACGGTTCGTTAAGGTATGTGG). The products of the IPCR were used in a second PCR, along with nested primers, to confirm that the correct fragment had been amplified. The PCR product was diluted 1:1000, and 2.5 μL of the product was used in the second PCR with primers E4 (5’-AACCGTAGAAAGGAAACCGT) and D73 (5’-CGGGATTTTCCCATCCTACTTTCATCCTG). A similar procedure was used to amplify the DNA adjacent to the 3’ end. Primers DL5 (5’-GGCTTAGTCAGTGAATACACGCGGCTG) and B39 (5’-TCGGTTTTATCCCCGATGATAATTAAAT) were used for the IPCR, and DL6 (5’-TTGGTCCGGAGATAACAGAGTCTAGC) and D71 (5’-CGTACGACCCTTTTTACCATCCA) were used as nested primers for PCR. The empty donor sites present after Ds(HYG 35s) excision were amplified using two TinY gene-specific primers, DWF1 (5’-TTTACACCCACCAACCC) and DWF2 (5’-GCTAGCTTCCAGCTTCTTG).

**Reverse Transcription-PCR**

RNA extractions were performed as described by Stiekema et al. (1988). Reverse transcription (RT)-PCR was performed by the method of Frohman et al. (1988). Reverse transcription was performed using the standard dT17 adapter primer (Frohman et al., 1988). Moloney murine leukemia virus (Gibco BRL), and 5 μg of total RNA. After the reaction, the mixture was diluted 10-fold, and 5 μL (out of 200 μL) was used directly for PCR in a total volume of 50 μL. The following primers were used for PCR. To detect the tiny transcript (Figure 10C), DWF3 (5’-AACCTGAGCAGTTGAGCG) and the standard dT17, adapter (Frohman et al., 1988) were used. To demonstrate the fusion between the Ds sequence and the tiny coding sequence (Figure 7C), D73 and the standard dT17, adapter (Frohman et al., 1988) were used.

**Isolation of tiny Revertant Alleles**

Plants that carried 35STPase and were heterozygous for both tiny and for the closely linked SPT gene, which is present within the T-DNA, were self-fertilized. Among the progeny, wild-type plants homozygous for the SPT gene were identified, and it was considered likely that these contained at least one revertant allele because they carried SPT on both chromosomes but tiny on neither of them. To identify these plants, families derived from plants heterozygous for both 35STPase and tiny were used to recover 150 phenotypically wild-type plants. These 150 plants were self-fertilized, and their progeny were grown on streptomycin-containing medium. All of the progeny of 13 of these 150 plants were streptomycin resistant, and therefore, these 13 wild-type plants were homozygous for the SPT gene. DNA from five of these plants, derived from two parental plants doubly heterozygous for tiny and 35STPase, were used for PCR and sequencing of the footprints. All fragments showed the same sequence (Figure 7).

**Scanning Electron Microscopy**

For the leaf cross-sections, leaves were mounted in Tissue-Tek (Agar Scientific Ltd., Stansted, Essex, UK) on an aluminium stub, plunged into nitrogen slush at -210°C for 30 sec to fix the tissue, and then transferred into the precooled prechamber of a scanning electron microscope (CamScan series IV; Gresham CamScan, Cambridge, UK).
The leaf was then fractured using a sharp scalpel blade and spattered with gold for 12 min. The specimen was viewed at an operating voltage of 16 kV while being held at −190°C on the Hexland cryo-stage (Oxford Instruments, Oxford, UK) of the scanning electron microscope. The hypocotyl and leaf epidermis were analyzed in the same way, except that the leaves were not fractured, and the hypocotyls were not plunged into nitrogen slush nor fractured.

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