A Gene Fusion at a Homeobox Locus: Alterations in Leaf Shape and Implications for Morphological Evolution

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Compound leaves are seen in many angiosperm genera and are thought to be either fundamentally different from simple leaves or elaborations of simple leaves. The knotted1-like homeobox (knox) genes are known to regulate plant development. When overexpressed in homologous or heterologous species, this family of genes can cause changes in leaf morphology, including excessive leaf compounding in tomato. We describe here an instance of a spontaneously arisen fusion between a gene encoding a metabolic enzyme and a homeodomain protein. We show that the fusion results in overexpression of the homeodomain protein and a change in morphology that approximates the changes caused by overexpression of the same gene under the control of the cauliflower mosaic virus 35S promoter in transgenic plants. Exon-shuffling events can account for the modularity of proteins. If the shuffled exons are associated with altered promoters, changes in gene expression patterns can result. Our results show that gene fusions of this nature can cause changes in expression patterns that lead to altered morphology. We suggest that such phenomena may have played a role in the evolution of form.

INTRODUCTION

The shoot apical meristem of a plant produces leaves in succession. Leaves are lateral organs that are determinate and bilaterally symmetrical and function in photosynthesis. Higher plants can have either simple or compound leaves. At a node on the stem, simple leaves have a single lamina that can sometimes be deeply lobed or dissected, whereas compound leaves elaborate numerous individual laminar units on a rachis that arises at a node. Simple and compound leaves often can be found in related species of the same genus (e.g., simple-leaved Solanum nigrum versus compound-leaved S. lycopersicoides). This implies that leaf form is plastic and that a compound leaf may be produced by progressive elaboration of a simple leaf, or a simple leaf by progressive simplification of a compound leaf. Researchers have proposed that the true nature of the compound leaf may be intermediate between that of an indeterminate shoot system and a determinate lateral organ (Sattler and Rutishauser, 1992). Genetic and molecular analyses focusing on model organisms such as Arabidopsis and maize have dealt only with simple leaves. In this study, we examine the genetic and molecular alterations that can change the nature of the compound leaf in tomato.

Homeodomain-containing transcription factors are known to play a role in the regulation of development in eukaryotes (McGinnis et al., 1984; Scott et al. 1989). This class of transcription factors is evolutionarily conserved among animals, plants, and fungi, and these genes may have diverged to assume distinct functions before these three kingdoms diverged (G. Bharathan, B.J. Janssen, E.A. Kellogg, and N. Sinha, submitted manuscript). The knotted1 (kn1)-like homeobox (knox) genes comprise two classes (Vollbrecht et al., 1991; Kerstetter et al., 1994). The class 1 genes include kn1, stm1 (shootmeristemless1), and knat1, and they are known to have a profound effect on vegetative development in plants. These genes are expressed specifically in cells with a stem cell-like fate at the shoot meristem and are absent from leaf primordia and leaves in simple-leaved plants (Jackson et al., 1994; Lincoln et al., 1994; J.A. Long et al., 1996). Overexpression of these genes under the control of a constitutive promoter in Arabidopsis or tobacco leads to lobed leaves with meristem proliferation (Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996). Based on these observations, researchers have proposed that this class of genes has a function in maintaining cells in an undifferentiated and meristematic state.

The cultivated tomato has a compound leaf, and the expression pattern of knox class 1 genes is somewhat different in tomato shoot apices. The tomato Tkn1 gene has been shown to be expressed in floral meristems but is also seen in developing leaf primordia. In addition, when the maize kn1 gene is overexpressed in tomato, an excessively proliferated compound leaf results (Hareven et al., 1996). Thus, the knox class 1 genes appear to confer meristem identity in species with simple leaves and leaflet proliferation in species with compound leaves (Sinha et al., 1993; Hareven et al., 1996).
However, the overexpression studies referred to above used the nonplant cauliflower mosaic virus (CaMV) 35S RNA promoter; their focus was to examine the effects of overexpression of heterologous genes (Sinha et al., 1993; Hareven et al., 1996).

Overexpression of kn1 and knat1 (both class 1 knox genes) has been compared in Arabidopsis. Overexpression of the maize gene kn1 produced phenotypes that in general were similar to those seen by overexpression of the Arabidopsis gene knat1 (Lincoln et al., 1994); however, a more detailed examination of the knat1 overexpression phenotypes revealed ectopic stipule and shoot meristem production in the leaf sinuses (Chuck et al., 1996). Homologous gene overexpression may reveal new information not observed in heterologous gene overexpression. The phylogenetic relationships of the various knox genes were also unclear at the time, making it difficult to interpret the evolutionary significance of the results. The homeobox-containing gene that we have characterized, LeT6 (for Lycopersicon esculentum T6; GenBank accession number AF000141), is a class 1 knox gene. Our results indicate that the LeT6 gene is orthologous to the Arabidopsis stml and soybean sbhl genes, whereas Tknl, or even Tknl, in tomato.

Cosegregates with and is Overexpressed in the Me Mutation

Wild-type tomato has a unipinnately compound leaf with a terminal leaflet and two or three pairs of major lateral leaflets with marginal lobes and pinnate venation (Figures 1A and 1D; Dengler, 1984). In addition, minor lateral leaflets occur between the major leaflets. Leaflets arise in basipetal succession from the adaxial region of the leaf primordium, and the leaf has a marked basipetal pattern of maturation (Dengler, 1984; N. Sinha, A. Williams, and N. Weber, unpublished data). This basipetal pattern of differentiation is marked by the presence of mature trichomes on the apex of young initiating leaf primordia. The Me mutation arose spontaneously in the isogenic tomato cultivar Rutgers (Rick and Harrison, 1959) and is a complete dominant. Me plants have leaves that are excessively proliferated so that the leaf is now three- or fourfold pinnate. In addition, leaflets are approximately cordate (heart shaped), lack marginal lobes, and have a palmate pattern of venation (Figures 1B and 1F). Me leaves are also larger than those of the wild type.

Me leaves often show numerous minute and underdeveloped leaflets on the rachis and at the bases of the major leaflets (Figure 1E). In addition, shoot primordia are sometimes seen to develop on the rachis of the leaves. In Me leaves, differentiation at the tip is delayed (as shown by the absence of trichomes on the tips on immature primordia), and the presumptive basal marginal lobe of the leaflets is precociously developed (Figure 1I). This leads to the heart shape on the leaflets (Figures 1B and 1F). Compared with that of the wild type, the Me leaf primordium delays production of leaflet primordia and shows precocious growth and development of the first pair of lateral leaflets and the basal marginal lobe (Figures 1H and 1I). A comparison of the shoot apical meristem between wild-type and Me plants shows that in an apex with four initiated leaf primordia (marked 1 to 4 in Figures 1H and 1I), multiple leaflet primordia have initiated on leaves 3 and 4 in the wild type, and that only one
The Me mutation is located on chromosome 2 (Rick and Harrison, 1959). We had previously identified and mapped tomato cDNAs encoding homeodomains and shown that one, LeT6, mapped to tomato chromosome 2 (B.-J. Janssen, A. Williams, J.-J. Chen, J. Mathern, S. Hake, and N. Sinha, submitted manuscript). LeT6 maps near Tg454 (lod score, 8.27). Tg454 is ~8.4 centimorgans (cM) distal from Prx-2,3 (confidence limit, 99%), which is also the genetic map distance between Prx-2,3 and Me. This indicated to us that LeT6 and Me are very closely linked on chromosome 2. To determine whether alterations at the LeT6 locus are involved in the Me mutation, we analyzed DNA from a segregating population of Me plants in the isogenic cultivar Rutgers background. DNA from individuals in the segregating population was digested, electrophoresed, blotted, and hybridized with the LeT6 cDNA probe. We detected a polymorphism in all mutants, and the polymorphism cosegregated with the Me phenotype in >100 individuals analyzed (Figure 1J), indicating that LeT6 and Me were within 1 cM of each other.

Our previous studies determined that LeT6 hybridized with an ~1600 base transcript that was expressed at high levels in shoot apical regions, floral buds, and immature ovaries and was undetectable in mature wild-type leaves (B.-J. Janssen, A. Williams, J.-J. Chen, J. Mathern, S. Hake, and N. Sinha, submitted manuscript). RNA gel blots were used to analyze LeT6 expression in Me leaves and leaves from the isogenic parental cultivar Rutgers. Our blot indicated that the LeT6 hybridizing transcript was indeed overexpressed in Me leaves (Figure 2A), whereas control probes indicated that Rutgers and Me RNA were equally loaded (Figure 2B). However, the transcript in Me was ~800 bases longer than the previously observed transcript in 35S-LeT6 transformed plants (Figure 2A) or wild-type buds.

To determine whether overexpression of the LeT6 cDNA in tomato under control of a constitutive promoter would lead to phenotypes similar to Me, we made CaMV 35S-LeT6 transformed plants and examined the leaf phenotypes produced. A majority of the transgenic plants showed excessive leaf compounding (Figure 1C). The leaf compounding was very severe in most cases, often leading to a leaf that could be up to 10-fold pinnate. The leaflets were rounded at the base and showed approximately palmate venation and lack of leaflet lobing (Figure 1G). In addition, numerous undeveloped leaflets were seen to occur on the rachis of mature leaves, which sometimes attained a size 10 times larger than that of the wild type. Such alterations in leaf shape and size were not described for 35S-knl overexpression in tomato (Hareven et al., 1996). These phenotypes were reminiscent of the Me leaf phenotype. RNA gel blots (Figure 2A) indicate that the LeT6 transcript accumulates to high levels in the leaves of the 35S-LeT6 transformants. These results suggest that overexpression of LeT6 could lead to the Me phenotype.

The Expression Domain of LeT6 Is Altered in Me

We performed in situ hybridizations with wild-type and Me shoot apices by using an antisense LeT6 probe (shown in Figure 3). In wild-type plants, there were high levels of LeT6 expression in the entire shoot apical meristem (Figures 3A and 3C) and axillary meristems (data not shown). Expression seemed reduced or absent from the L1 layer of the shoot apical meristem (Figures 3A and 3C), as has been noted for knl, and may be an indication that just like knl, the LeT6 protein is capable of trafficking through plasmodesmata (Lucas et al., 1995). Examination of serial sections through entire shoot apices revealed that LeT6 expression was somewhat reduced compared with the shoot apical meristem but consistently present above background levels in the surrounding developing leaf primordia in wild-type plants and in the adaxial regions in the middle of the older leaf primordia. LeT6 expression was also seen in leaflet primordia and developing leaflet margins (Figures 3A and 3C). This expression pattern is in sharp contrast to that seen for knl in maize apices and stm1 and knat1 in Arabidopsis apices. In both of these species, the expression of the class 1 knox genes was absent from a region in the shoot apex representing P0, the next leaf primordium that forms. In addition, expression was not seen in immature leaf primordia (Jackson et al., 1994; Lincoln et al., 1994; J.A. Long et al., 1996). In tomato, the P0 primordium does not appear to be demarcated by absence of LeT6 gene expression. This may have important consequences on the type of leaf that develops from the primordium. Leaves are compound in tomato and simple in maize and Arabidopsis.

We also analyzed the pattern of LeT6 mRNA expression in homozygous Me apices (Figures 3B and 3D). The apices showed a much more expanded domain of LeT6 expression. In addition to high levels of expression throughout the shoot apical meristem, the LeT6 mRNA was highly localized in initiating leaf primordia and in the regions basal to the shoot apex in a cylinder that extended around the central vasculature. Expression was also seen in the axillary meristem in Me apices (Figures 3B and 3D). The LeT6 transcript was expressed at higher levels in the terminal region of initiating and more mature leaves in Me plants, with elevated expression in the adaxial region of leaves, as was seen in the wild type. These expression patterns correlate well with what we know of the Me phenotype. The terminal region of Me leaves matures late, and high adaxial expression marks...
Figure 1. Phenotypic Comparison of Wild-Type, Me, and 35S-LeT6 Plants.
A Hybrid Transcript in Me Contains LeT6 and Phosphofructokinase Sequences

We used polymerase chain reaction (PCR) products specific to the 5' and 3' end of LeT6 to sequentially probe RNA gel blots. The wild-type LeT6 is ~1600 bp in length. The 5' probe spanned 288 bp, which included 102 bases of untranslated leader and 186 bases of coding sequence. The 3' probe spanned 354 bp starting at base 979 and included the homeobox region. Using the 3' probe, we detected a hybridizing transcript in Me leaves and wild-type buds (Figure 4A). However, using the 5' probe, we detected no hybridizing transcript in Me leaves (Figure 4B). Hybridization with a rDNA control probe was used to confirm the presence of RNA in all lanes (Figure 4C). These results suggest that the hybridizing transcript in Me is very similar to the LeT6 cDNA in the 3' region but may have unknown sequences in the 5' region.

Figure 1. (continued).

(A) and (D) A mature wild-type (cv Rutgers) unipinnate leaf showing a terminal leaflet and three pairs of lateral leaflets. Smaller leaflets are visible between the lateral leaflets. The boxed region is enlarged in (D) and shows serrated leaflet margins and pinnate venation in leaflets.

(B), (E), and (F) A mature Me leaf showing numerous orders of lateral leaflets. The boxed region in (B) is enlarged in (E) and shows smooth leaflet margins and immature leaflets (white arrow) borne at the base of the mature leaflets. (F) is an enlargement of Me leaflets showing a pinnate venation pattern and a rounded leaflet base region.

(C) and (G) A mature 35S-LeT6 leaf showing several orders of lateral leaflets. The boxed region is enlarged in (G) and shows smooth leaflet margins, rounded bases, and palmate venation in the leaflets. Numerous immature leaflets can be seen among the more mature leaflets.

(H) Scanning electron microscopy of a wild-type shoot apex showing the shoot atypical meristem (m), four initiated leaf primordia (labeled from youngest to oldest as 1 to 4), and initiating leaflet primordia (LL) on leaves 3 and 4. The white arrow points to an initiating trichome at the tip of leaf 2. Bar = 100 μm.

(I) Scanning electron microscopy of an Me shoot apex showing the shoot apical meristem (m), four initiated leaf primordia (labeled from youngest to oldest as 1 to 4), and initiating leaflet primordia (LL, small arrow) on leaf 4. The initiating leaf lobe on leaf 4 is marked with a large arrow. The white arrow points to an initiating trichome in the middle of leaf 2. The tip of leaf 2 shows no trichomes. Bar = 100 μm.

(J) DNA prepared from Me (homozygous and heterozygous individuals) and L. esculentum cv Rutgers (wild-type) tissue from a segregating population, restricted with endonuclease HindIII, electrophoresed on a 1% agarose gel, and blotted to a membrane. The membrane was hybridized with an LeT6 cDNA probe. DNA from Me and wild-type cv Rutgers plants is marked in the appropriate lanes. The 8.5-kb band is seen in all individuals, the 7.3-kb band is seen in wild-type and Me heterozygotes, and the 6.8-kb band is seen only in the Me individuals.

To determine the nature of the LeT6-hybridizing RNA species in Me, we constructed a cDNA library in λOCUS with mRNA extracted from Me leaves and hybridized the 3' LeT6 probe with this library under high-stringency conditions. A total of 14 hybridizing clones was purified from an unamplified primary library containing ~10^6 clones. The identity of the region of the leaf that later proliferates to make lateral leaf primordia. It is evident that there is early complexity of the wild-type leaf primordium, whereas the primordia remain relatively undifferentiated at similar developmental stage in the Me shoot apex (compare Figures 1H and 1I to Figures 3A and 3C, and 3B and 3D, respectively). Using the entire LeT6 cDNA (including the homeobox region), we detected only one hybridizing transcript in Me leaves and wild-type bud tissue on RNA gel blots. Our in situ hybridization conditions were at a stringency equal to or greater than that used for the RNA gel blot analysis, indicating that we are only observing expression of LeT6-hybridizing RNA. This RNA is expressed at higher levels in initiating leaf primordia and in a more expanded domain in the Me apex compared with the wild type.

Figure 2. Gel Blot Analysis of RNA from Mature Leaves of Wild-Type (cv Rutgers), Me, and 35S-LeT6 Plants.

(A) Total RNA from Me, wild-type cultivar Rutgers (Ru), and two independent 35S-LeT6 plants was electrophoresed, blotted, and hybridized to a full-length LeT6 cDNA probe. No hybridizing RNA could be detected in wild-type leaves. The hybridizing RNA is 1.6 kb in 35S-LeT6 leaves and 2.4 kb in Me leaves.

(B) The same blot shown in (A) was stripped and rehybridized with a control (plastocyanin) probe. The length of the marker at right is given in kilobases.
Figure 3. In Situ Localization of the LeT6 Transcript in Wild-Type and Me Shoot Apices.
the clones was confirmed by PCR amplification of a DNA segment spanning the homeobox region, using LeT6-specific primers. Four of the 14 clones were longer than 1 kb and were analyzed further by sequencing.

In the 5' region of each of these four cDNAs, we found a sequence unrelated to LeT6, whereas the 3' region was identical to LeT6. A search of the databases revealed that this 5' region had 98% amino acid similarity to potato β subunit of PPI-dependent phosphofructokinase (PFP; GenBank accession number M55191). The PFP enzyme has been studied extensively in several organisms and catalyzes a near-reversible reaction that responds to changes in aspects of metabolism in a very flexible manner (Stitt, 1990).

The four cDNA clones fell into two classes, and all four had unique 5' ends, indicating that they represented individual transcripts. The 3' end of the fusion was identical in both classes, but the LeT6 ends were different. In the first class of cDNAs (PFP-LeT6-1), identical PFP sequences were fused at base 498 of LeT6. The predicted protein from PFP-LeT6-1 has the LeT6 homeodomain in frame with the predicted PFP protein (Figure 4D). PFP-LeT6-1 fusion includes the ELK domain (Vollbrecht et al., 1991) that contains the nuclear localization signal in Kn1 (Meisel and Lam, 1996) and presumably all class 1 and 2 knox genes. Although this gene fusion is missing 64 amino acids at the N-terminal end of LeT6, all features conserved in class 1 KNOX proteins (B.-J. Janssen, A. Williams, J.-J. Chen, J. Mathern, S. Hake, and N. Sinha, submitted manuscript) remain intact. Two of the four long cDNAs analyzed belonged to this class. In the second class of cDNAs (PFP-LeT6-2), identical PFP sequences were fused to base 659 in LeT6. The two other long cDNAs were of this second type. The predicted protein from PFP-LeT6-2 did not have the LeT6 homeodomain in frame with PFP, and a stop codon (TAG) was present 23 amino acids into the LeT6 sequence (Figure 4D). These amino acids are unrelated to the predicted LeT6 protein.

The β subunit of PFP is thought to be the catalytic part of the PFP enzyme (Yan and Tao, 1984). In both fusion events, all residues that are conserved between known PFP β subunit molecules, as well as residues involved in the binding of substrate, ATP, and effector molecules, appeared to be intact. However, we do not know whether the PFP part of the fusion protein remained fully functional.

Because only one of the fusion cDNAs (PFP-LeT6-1) can lead to the production of a protein with a homeodomain, we used reverse transcriptase-PCR (RT-PCR) to estimate the relative steady state levels of each kind of transcript. Our results suggest that PFP-LeT6-1 is present in excess over PFP-LeT6-2 (data not shown). It is likely that the out-of-frame transcript is degraded via a nonsense-mediated transcript decay pathway (Voelker et al., 1990; Caponigro and Parker, 1996; Ross, 1996; Van Hoot and Green, 1996). However, we saw an equal ratio of the two transcripts represented in cDNA clones. This discrepancy may be due to loss of one of the RT-PCR primer binding sites early in the degradation process. Alternatively, because only four of 14 clones were long enough to show the PFP-LeT6 junction, this equal ratio of the two different cDNAs could also be coincidental.

A Duplicated Copy of PFP Is Fused to the LeT6 Locus in Me

DNA blot analysis was used to characterize the LeT6 and PFP loci in Me and its isogenic parental line Rutgers. Genomic DNA from Me and cultivar Rutgers was digested with a range of restriction enzymes, electrophoresed, blotted, and first hybridized with a full-length LeT6 cDNA clone (Figure 5A) and then stripped and rehybridized with a probe made from the PFP region of the Me cDNA clone (Figure 5B). The results shown from blots using the LeT6 probe indicate that LeT6 is a single-copy gene in both cultivar Rutgers and Me plants and that fragments corresponding to the 5' end of the LeT6 gene are polymorphic in the Me mutation (as determined by DNA blot analysis using 5' and 3' probes; data not shown). For example, in Figure 5A, the EcoRI digest of Rutgers (lane 2) contains two bands (resulting from digestion of a single EcoRI site in the second intron of the LeT6 gene). The band at 9 kb (lower) corresponds to the 3' end of the LeT6 gene. This band is unaltered in Me plants (lane 1).

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Figure 3. (continued).

(A) and (C) Longitudinal sections of wild-type shoot apices were hybridized with an LeT6 antisense riboprobe. The hybridization signal is visible as silver grains on the tissue. The section in (A) is median; the section in (C) is on the flank of the shoot apical meristem. The meristem (m) shows a strong signal. Signal is also visible in the initiating leaf primordia (Lp), the initiating leaflet primordia (LL), vascular tissues (v), and at the edges of the leaflet blade (arrowhead in [A]). The section in (C) on the flank of the shoot apical meristem shows a leaf base (LB) of a more mature leaf with reduced signal. Because the initiating leaf primordia become complex very early (see Figure 1H), leaflet primordia are seen in every section. (B) and (D) Longitudinal sections of Me shoot apices were hybridized with an LeT6 antisense riboprobe. The hybridization signal is visible as silver grains on the tissue. The section in (B) is median. The section in (D) is on the flank of the shoot apical meristem. The meristem (m) shows a strong signal. Signal is also visible in a cylinder encircling the central region of the axis, the developing axillary bud (a), and the initiating leaf primordia (LP). A strong hybridization signal is also seen at the tip of the leaf primordium and adaxial region of the developing tip (arrowheads in [B] and [D]). Because the initiating leaf primordia do not show early complexity (see Figure 1H), leaflet primordia are not seen in these Me sections. (A) to (D) represent adjacent photographs compiled from the same tissue section.
The Plant Cell

Figure 4. RNA Gel Blot Analysis of Various Tissue Samples from Wild-Type (cv Rutgers) and Me Plants.

(A) Total RNA was fractionated by gel electrophoresis, blotted onto a nylon membrane, and hybridized with a LeT6 3' specific probe containing homeobox sequences. The region covered by the probe is given in (D). A 2.4-kb RNA hybridized with the Me leaf sample. The hybridizing transcript was 1.6 kb in wild-type shoot tip RNA. Mature wild-type leaf and immature wild-type seed RNA showed no hybridization. wt, wild type.

(B) The same blot as shown in (A) was stripped and rehybridized with an LeT6 5' probe. The region covered by the probe is given in (D). The Me leaf, mature wild-type leaf, and immature wild-type seed RNA showed no hybridization. The hybridizing transcript was 1.6 kb in wild-type shoot tip RNA.

(C) The same blot as shown in (A) was stripped and rehybridized with an 18S rDNA probe. The length marker is given at right in kilobases.

(D) Shown is a diagram of the structure of the PFP β-LeT6 fusion cDNAs showing the L. esculentum cv Rutgers cDNA for reference. The regions used as hybridization probes on RNA gel blots are shown. Only the 3' portion of the PFP β sequence is shown in each case. The homeobox region in the LeT6 or LeT7 part of the fusion cDNA is indicated by crosshatching. Lines indicate exon–intron boundaries; numbers indicate the start of transcription of LeT6. Dashed lines indicate that not all of LeT6 and PFP is included in the diagram. ATG, start codon of LeT6 coding region; Ex, exon; TAG, stop codon in PFP–LeT6–2; TGA, LeT6 stop codon.

The upper band (10 kb) in Rutgers plants corresponding to the 5' end of the LeT6 gene is shifted to ~12 kb in Me plants. Hence, the 5' end of the LeT6 gene has been altered in the Me mutation.

DNA blot hybridization with a PFP probe showed an additional copy of the PFP locus in Me DNA. In addition, in each case in which an additional band was observed using the PFP probe, the new band comigrated with the polymorphic band that was detected by using the LeT6 probe. For example, in Figure 5B, the EcoRI digest of Rutgers (lane 2) contains a single band at ~15 kb. When Me DNA was digested with EcoRI (lane 1), two bands could be seen: the ~15-kb band and a new band at 12 kb that migrated at the same position as the polymorphic LeT6 band seen in Figure 5A. A similar result was obtained when Rutgers and Me DNA were double digested with HindIII and EcoRI or digested with HindIII alone. Using the LeT6 probe, we observed at 7 kb in Me a polymorphic band corresponding to the 5' end of the LeT6 gene (Figure 5A, lanes 3 and 5). Using the PFP probe, we detected a new band at 7 kb (Figure 5B, lanes 3 and 5) in addition to the band seen at 6.5 kb in both Me and Rutgers DNA (Figure 5B, lanes 3 to 6).

The presence of a polymorphic band corresponding to the 5' end of the LeT6 gene in Me DNA combined with the presence of a new PFP hybridizing band that comigrated with the polymorphic LeT6 band suggests that a portion of the PFP locus has been duplicated in Me and that this duplicated region is fused to the 5' end of the LeT6 gene.

To determine whether the fusion cDNAs represented products of splicing events, we determined the intron–exon boundaries in LeT6 by using a PCR-based approach. Regions of both the cDNA and genomic DNA were amplified using LeT6-specific primers, and the PCR products were analyzed for size differences. The results obtained from this analysis were also confirmed by using a partial genomic clone for LeT6 from cultivar VF36 (L. Lund, B.-J. Janssen, and N. Sinha, unpublished data). The structure of the LeT6 coding region is presented graphically in Figure 5D.

We used a PCR-based approach to isolate and characterize the genomic region that spans the fusion between the LeT6 and PFP genes. A cDNA clone of PFP was isolated from a VFNT Cherry young fruit library (cv VFNT Cherry LA 1221, as described by Narita and Gruissem [1989]) and aligned with the Ricinus communis genomic sequence (GenBank accession number Z32850) to identify exons. Primers were designed in exon 13 (PFP1) and exon 15 (PFP5) from the tomato cDNA sequence. Genomic DNA from cultivar Rutgers was amplified using these PFP-specific primers, cloned, and sequenced (Figure 5D). The 5' region of a partial genomic clone of LeT6 from cultivar VF36 was also sequenced (Figure 5D). The region of genomic DNA from Me containing the fusion between LeT6 and PFP was amplified using primer PFP1 and a primer in exon 1 of LeT6. A single 2.4-kb fragment was amplified, cloned, and sequenced (Figure 5D).

An alignment of the Me sequence with that obtained from the VF36 LeT6 5' region and the Rutgers PFP gene is shown in
The Me fragment contained the 5' region of LeT6 and 1630 bp of the upstream region from the LeT6 gene fused directly to intron 14 of the PFP gene. At the fusion point, there are 4 bp of local sequence homology between the LeT6 and PFP sequences that may have played a role in the duplication event.

The locations for introns 13 and 14 were identical between PFP from R. communis and tomato, although the size and sequence of the introns we compared were variable (52 and 49% nucleotide identity for introns 13 and 14, respectively).

Over exons 13, 14, and 15 of PFP, the nucleotide sequences in R. communis and tomato cultivar Rutgers have 82% identity, and the amino acid sequences are 95% identical. Therefore, we used the R. communis genomic organization to infer exon–intron boundaries for tomato PFP. DNA gel blot analysis of Me and Rutgers, using five different restriction enzymes and hybridization with a PFP probe, indicated that at least 15 kb of genomic DNA upstream of the fusion junction are identical in the duplicated and normal versions of PFP (four of these digests are shown in Figure 5C). The PFP-hybridizing fragment is twice as intense in Me as in Rutgers, consistent with the presence of a duplicated copy of PFP in Me (Figure 5C). Based on comparisons with the R. communis gene, the duplicated PFP locus in the Me PFP–LeT6 fusion has ~10 kb of native PFP upstream sequences.

We also hybridized RNA gel blots containing RNA from mature leaves of wild-type and Me plants with a PFP-specific probe to determine the levels of PFP transcripts in each leaf type (Figure 5E). In mature wild-type leaves, PFP expression (2-kb hybridizing transcript) was low. In Me leaves, two hybridizing RNA species were detected. One was ~2.4 kb, which also hybridized with the LeT6 probe and represented the PFP–LeT6 fusion transcripts. The second transcript was ~2 kb, which did not hybridize with the LeT6 probe, and represented the complete PFP β subunit mRNA. The low abundance of the PFP–LeT6–2 transcript, variation in poly(A) tail lengths, and inadequate resolution on the gels could account for the absence of a third transcript on our RNA gel blots. In RNA from mature Me leaves, the PFP β subunit transcript was present at levels higher than those seen in wild-type leaf RNA. We also hybridized RNA gel blots from mature 3S–LeT6 leaves. The levels of PFP β subunit mRNA were higher in these leaves compared with those in the wild type (data not shown). This may indicate that the levels of PFP β subunit mRNA are somehow upregulated by high levels of LeT6. A 2-kb PFP hybridizing transcript was also abundant in RNA from wild-type seeds and bud tissue. The presence of normal PFP transcript in Me leaves confirms that in Me, the PFP locus is duplicated.

Elevated LeT6 Levels in Tomato Leaves Lead to Extra Compounding

We have compared leaf phenotypes resulting from a spontaneously arisen and an engineered overexpression of LeT6. In 3S–LeT6-transformed tomato plants, leaves with excessive compounding are produced. The leaflets are approximately heart shaped, show no marginal lobing, and have palmate venation. The characteristic feature of these transgenic leaves is that they produce lower order leaflets from the bases of more mature leaflets and from the rachis. Unlike wild-type mature leaves that have undetectable LeT6 expression, all transgenic plants with excessive leaf compounding show high levels of the LeT6 transcript in mature leaves. This feature also appears to delay the senescence of these leaves. We have seen transgenic plants with leaves persisting for ~6 months. With PFP–LeT6 overexpression in Me, most of the features mentioned above are seen in leaves. Leaflets lack lobes, are heart shaped, and have palmate venation. In addition, leaflets can proliferate from the rachis, and leaves have delayed senescence.

The similarity between the two phenotypes indicates that the LeT6 portion of the fusion product most likely provides the phenotypically functional domain of the protein. If there are any alterations in the binding specificity of the LeT6 homeodomain in the PFP–LeT6–1 fusion product, they appear to be minor. The portion of LeT6 protein deleted from the PFP–LeT6–1 fusion leaves intact all features conserved between the class 1 KNOX proteins (B.-J. Janssen, A. Williams, J.-J. Chen, J. Mathern, S. Hake, and N. Sinha, submitted manuscript), including the nuclear localization signal (Meisel...
Figure 5. DNA Blot Analysis of the PFP and LeT6 Loci.
and Lam, 1996). Recent domain-swapping experiments for KNOX proteins indicate that functional specificity resides in the ELK and homeodomain regions (Sericikawa and Zambryski, 1997). Because these regions are present at the C terminus in both LeT6 and PFP-LeT6, they are likely still to be able to find the appropriate promoter sequences and to bind to them and also to interact with other proteins of the transcription complex.

Our results indicate that the PFP and LeT6 genes and the Me mutation map coincident with each other on chromosome 2. The Me mutation arose spontaneously in the isogenic line Rutgers. Restriction fragment length polymorphism analysis with LeT6 placed LeT6 within 1 cM of Me. DNA gel blot analysis and cloning of the locus indicate that the PFP gene is duplicated and fused to the 5’ region of LeT6. RNA gel blots and cDNA cloning showed that a PFP–LeT6 fusion transcript is overexpressed in mature leaves from Me. The LeT6-hybridizing transcript was expressed in an enlarged domain and at higher levels in shoot apices of Me plants, and altered expression patterns in Me correlated well with the mutant phenotype. Overexpression of LeT6 in tomato leads to phenotypes that resemble the Me mutation. These data offer compelling arguments that a gene fusion between PFP and LeT6 is the primary cause of the Me mutation.

**LeT6 Fusion to PFP Leads to Altered Expression Levels and Patterns**

Overexpression of LeT6 in wild-type tomato leaves under the control of the CaMV 35S promoter caused the production of a highly compound leaf with altered leaf shapes. The CaMV 35S promoter is known to give rise to high and ubiquitous levels of gene expression in plants (Bentley and Chua, 1990). Because high levels of LeT6 expression (35S–LeT6) conferred excessive compounding on leaves and the altered leaf phenotypes in PFP–LeT6 were very similar to the 35S–LeT6 leaf phenotypes, we infer that elevated levels of the LeT6 portion of PFP–LeT6-1 are responsible for excessive compounding in Me leaves.

We observed a significant rise in PFP–LeT6 expression levels in the leaf primordia in Me when compared with that of the wild type. LeT6 expression in Me apices compared with that in the wild type was altered in the domain of PFP–LeT6 expression. These apices showed elevated expression in the adaxial and tip region of the leaf primordium, higher levels of mRNA expression in initiating leaf primordia, and a more expanded domain of expression in the shoot apical meristem itself. In wild-type leaf primordia, leaflets are produced from the adaxial region of the primordium. The presence of LeT6 RNA in the region indicates that expression in the adaxial domain in immature leaves plays a role in leaf compounding in tomato. Higher levels of expression in the tips of initiating leaves correlate with the delayed maturity of these leaf tips in Me plants. In addition, higher overall expression of PFP–LeT6 in the leaf primordium can account for extra leaflet proliferation in these leaves.

**Role of Class 1 knox Genes in Leaf Initiation and Development**

The class 1 knox genes are known to play a role in shoot meristem organization and maintenance in maize and Arabidopsis (Smith et al., 1992; Sinha et al., 1993; J.A. Long et al., 1996). Ectopic overexpression of these genes in both maize and Arabidopsis leads to abnormalities in leaf shape, size, and growth patterns (Smith et al., 1992; Lincoln et al., 1994; Sinha and Hake, 1994; Chuck et al., 1996). In addition, ectopic shoot meristems are often produced on these
abnormal leaves (Sinha et al., 1993; Chuck et al., 1996). It has been suggested that the primary role of the class 1 knox genes may be to maintain indeterminate cell fates, and their role, if any, in leaf development may be minor. Downregulation of knox genes in the P subdomains has been considered a hallmark for leaf initiation in maize and Arabidopsis (Smith et al., 1992; Jackson et al., 1994; Lincoln et al., 1994). When 35S-kn1 is overexpressed in tobacco (possessing simple leaves), leaf shape, size, and determinacy are altered (Sinha et al., 1993), but the leaf produced remains simple. When expression of KN1-like proteins is analyzed in 35S-kn1 tobacco shoot apices, there appears to be a downregulation of KN1-like proteins in the P region, but ectopic expression (presumably of the 35S-kn1 transgene) is still seen in the developing leaf primordia (D. Jackson and S. Hake, personal communication). Thus, even under synthetic overexpression conditions using strong promoters, downregulation of knox gene expression is seen in the P region of simple-leaved tobacco.

The Arabidopsis stm gene, which is orthologous to LeT6 (G. Bharathan, B.-J. Janssen, E. Kellogg, and N. Sinha, submitted manuscript), is also shown to be absent from the P region of leaf and floral organ primordia (J.A. Long et al., 1996). In contrast, the LeT6 homeobox gene is expressed throughout wild-type shoot apical meristems and in immature leaves in tobacco. Downregulation in P develops leaf primordia (this study) or floral primordia (B.-J. Janssen, A. Williams, J.-J. Chen, J. Mathern, S. Hake, and N. Sinha, submitted manuscript) was not observed for LeT6, indicating that global alterations in expression patterns of LeT6 may have occurred in tomato. Recently, kn1 overexpression has been analyzed in 35S-kn1 tobacco shoot apices, there appears to be a downregulation of KN1-like proteins in the P subdomains, but ectopic expression (presumably of the 35S-kn1 transgene) is still seen in the developing leaf primordia (D. Jackson and S. Hake, personal communication). Thus, even under synthetic overexpression conditions using strong promoters, downregulation of knox gene expression is seen in the P region of simple-leaved tobacco.

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Evolutionary Implications of Gene Fusions

The modular nature of eukaryotic genes seems to be well recognized (Doolittle, 1995). It has been suggested that these modules were moved around to give rise to modern genes by using the process termed exon shuffling (Gilbert, 1978; M. Long et al., 1995, 1996). Several examples of modularity have recently been seen in disease resistance genes in plants (Martin et al., 1993; Whitham et al., 1994; Song et al., 1995). However, no mechanism has been proposed to explain how these modules can be shuffled. Our results demonstrate one mechanism by which exons can be shuffled and modules combined. In the Me mutation, a duplicated version of the PFP β subunit is fused to a homeobox-containing gene LeT6. The break point is in intron 14 of PFP, and this causes transcription through ~1.6 kb of the LeT6 promoter and the LeT6 coding region. The primary transcript is then spliced in one of two ways. One joins exon 14 of PFP to a cryptic site within exon 1 of LeT6, and the other joins exon 14 of PFP to the second exon of LeT6.

Such gene fusions have been described in the biology of human cancers. In the TPR-MET fusion, translocation of TPR (for translocated promoter region) to the MET protooncogene (hepatocyte growth factor receptor, which is a tyrosine kinase) results in downregulation of kinase activity and mimics ligand binding to lead to an oncogenic response (Santoro et al., 1996). Similarly, in the BCR-ABL fusion, a translocation causes fusion of BCR (for break cluster region) to ABL (for avian blastic leukemia) and results in increased tyrosine kinase activity leading to myelogenous leukemia (Glasman, 1995). In the E2A-PBX gene fusion, the transcriptional activation domains from E2A (transcription factors with basic helix-loop-helix motifs regulating cell type-specific genes in B-lineage lymphoid cells) are fused to the PBX gene (a divergent homeobox gene serving a general role as a transcription factor in all but lymphoid cells). In this fusion, the basic helix-loop-helix region of E2A is lacking, but presumably the regulation and transcriptional activation domains of E2A are fused to the homeodomain of PBX. This causes PBX-regulated genes to be activated/repressed in regions in which PBX is not normally located (LeBrun and Cleary, 1994). These examples of gene fusion appear to play a role in cell division and differentiation but not in morphogenesis per se.

Similar gene fusion events occurring in important regulatory genes (or leading to early expression patterns that can alter morphogenesis) may have been important in the mor-
phological evolution of both plants and animals. Fusions like the one we report here can explain several phenomena. They can account for exon shuffling and the addition of modules to proteins during evolution. They can account for the presence of regulatory control elements seen in certain introns. The residual LeT6 promoter in PFP-LeT6 functions as an intron, and any transcription enhancement or repression features that are present in it may also regulate the fusion transcript. Most important of all, this type of gene fusion event can explain how the expression pattern of a protein involved in morphogenesis may be changed in a single step to lead to a dramatic alteration in phenotype. Whereas base pair changes in coding sequences occur over time, they are often not sufficient to account for phenotypic evolution. Major alterations in promoter regions of key genes can provide an explanation for morphological changes. Relatives of the cultivated tomato show leaves that are either more or less compound than those seen in tomato. Gene fusion and exon capture events like the one we describe can lead to dramatic changes in the degree of leaf compounding. Such changes in the evolutionary history of tomato and its relatives could explain the great variation in leaf form that exists in the tomato family.

METHODS

Histology and Scanning Electron Microscopy

For histological studies, fresh or fixed leaf tissue (FAA; Berlyn and Miksche, 1976) was sandwiched in pith sticks and hand sectioned using razor blades, mounted in water or glycerin, and observed under visible light. Photographs were taken using 160 Ektachrome film or PanX film (Eastman Kodak, Rochester, NY). For thin-section examinations (8 to 10 μm), 1 cm² of leaf tissue was fixed in freshly made FAA for 30 min at room temperature, then dehydrated through a graded ethanol series, equilibrated in Histoclear (National Diagnostics, Atlanta, GA), and gradually embedded in Paraplast (Fisher Scientific, Pittsburgh, PA) over a period of 2 days. Tissue blocks were cast, and 8- to 10-μm sections were cut using a metal knife (Fisher Scientific) on a rotary microtome (Bausch and Lomb, Rochester, NY). Tissue ribbons were mounted in water on ProbeOn Plus slides (Fisher Scientific) and kept at 42°C overnight. ProbeOn Plus slides were paraffinized in Histoclear, rehydrated through a graded ethanol series, and treated for various in situ localization methods. Slides were then dehydrated in ethanol, cleared in Histoclear, and mounted in Permount (Fisher Scientific) for examination.

For scanning electron microscopy, fresh tissue was fixed overnight in 3% glutaraldehyde in phosphate buffer, pH 7.2 to 7.4 (O'Brien and McCully, 1981), dehydrated in a graded ascending ethanol series, and critical point dried with CO₂. Samples were mounted on scanning electron microscopy stubs with epoxy or carbon-coated tape, sputter coated with a 25-nm layer of gold or gold-palladium in a sputter coater, and then viewed with an Amray 100 (Amray, Bedford, MA) or a JEOl JSM-6100 (JEOL USA, Peabody, MA) scanning electron microscope running at an accelerating voltage of 5 to 20 kV. Photography was performed with Polaroid 55 film (Eastman Kodak) directly from the scanning electron microscope.

RNA and DNA Gel Blots

Fresh tomato tissue was collected and frozen in liquid nitrogen. Total RNA was extracted by the hot phenol method. Briefly, buffer-equilibrated phenol was mixed in equal proportions with extraction buffer (100 mM LiCl, 100 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS). To this mixture, which was kept in a heat block at 80°C, was added tissue ground to a fine powder in liquid nitrogen. The tubes were vortexed in a fume hood, and half of the volume of chloroform was added to each tube. The aqueous phase was separated by centrifugation and precipitated with an equal volume of 4 M LiCl at −20°C. The precipitated RNA was further purified using standard ethanol precipitation. RNA samples were resolved on a 1% formaldehyde gel, transferred to Hybond N+ charged membranes (Amersham), and hybridized as described by Sambrook et al. (1989). The LeT6 3'-specific probe (see Figure 4A) was a polymerase chain reaction (PCR) product (primer JT6, 5'-TCATCGACCCCCAGGCTG-3'; primer NST1, 5'-AAATGTTAGCAAGAAGATT-3'), as was the 5'-specific probe (primer NST2, 5'-TITITICTAGTGTGTTGTGA-3'; primer JT5, 5'-CTTCTTATTGTATTATTGTTT-3). After hybridization, filters were washed twice in 0.1% SDS at 65°C for 1 hr. Under these conditions, hybridization temperatures were 15°C below the melting temperature, and the washes were done at 5 to 7°C above melting temperature (melting temperature Tm calculations were done according to Sambrook et al. [1989]). The phosphofructokinase (PFP)-specific probe was derived from the PFP-LeT6-1 cDNA by digestion with EcoRI and SacI and gel purification of the 344-bp PFP-specific fragment. The LeT6 probe was a PCR fragment containing the entire LeT6 cDNA and amplified using T3 and T7 primers. Loading of RNA was estimated by hybridization with a labeled Arabidopsis rDNA probe containing 18S sequences (Prutt and Meyerowitz, 1986) or a cDNA clone of the tomato plastocyanin gene (kindly provided by N. Hoffman, Carnegie Institute of Washington, Stanford, CA). Probes were labeled with phosphorus-32, using the Promega Prime-a-Gene labeling system, according to the manufacturer's instructions.

DNA was extracted essentially using the method of Dellaporta et al. (1983). Briefly, ~1 g of frozen tissue was ground to a fine powder under liquid nitrogen and added to 10 mL of extraction buffer (100 mM Tris·HCl, pH 8.0, 50 mM EDTA, and 500 mM NaCl) containing 40 μL of 2-mercaptopethanol. SDS (1 mL of a 20% solution) was added, and the mixture was incubated at 65°C for 10 min. Precalched 5 M potassium acetate (10 mL) was added, and the samples were mixed, incubated on ice for 20 min, and then centrifuged at 16,000g for 20 min at 4°C. The supernatant was passed through cheesecloth, and the DNA was precipitated by the addition of 10 mL of 2-propanol and incubation at 4°C for 1 hr. DNA was pelleted by centrifugation at 16,000g for 20 min at 4°C, rinsed twice with 70% ethanol, air dried, and resuspended in 400 μL of water containing 20 μg/mL RNase A. When necessary, DNA was further purified by phenol-chloroform extraction and precipitation.

For DNA gel blots, genomic DNA (20 μg) was digested with restriction enzymes in a large volume (400 μL) and then ethanol precipitated, rinsed with 70% ethanol, air dried, resuspended in water, and separated on a 0.8% agarose gel. After electrophoresis in 0.5× TBE (22.5 mM Tris·HCl, pH 7.6, 22.5 mM boric acid, and 0.5 mM EDTA) at 5 V/cm, the gel was stained with ethidium bromide and photographed.
depurinated in 0.25 M HCl, denatured in 0.4 M NaOH and 0.6 M NaCl, and transferred to a Hybond N+ charged nylon membrane using capillary transfer overnight in 0.4 M NaOH and 0.6 M NaCl. Hybridization was performed essentially as described by Sambrook et al. (1989), using the following hybridization buffer (50 mM Pipes, 50 mM sodium phosphate, pH 7.0, 100 mM NaCl, 1 mM EDTA, and 5% SDS). After hybridization, membranes were washed four times in 0.5 × SSC (SSC made according to Sambrook et al. [1989]) and 0.1% SDS at 65°C for 20 min and autoradiographed.

cDNA Library Construction and Screening

Poly(A)+RNA was isolated by using the Poly-A-Tract mRNA isolation system III (Promega). The Mouse ears (Me) mature leaf cDNA library was constructed in the λGUS vector, using a directional cDNA library construction system (Novagen, Madison, WI). The primary library had a titer of ~109 plaque-forming units. The library was screened using a random primer labeled (Prime-a-Gene) PCR fragment from the 3' end of the LeT6 cDNA (primers NST1, 5'-AAATGGTTAGCCAAGACATT-3'; and JT6, 5'-TCATCGACCCCCAGGCTG-3'). Plasmid DNA was prepared by the suggested CRE/LOX-mediated excision protocol (Novagen).

A cDNA clone of PFP was isolated from a cultivar VFNT Cherry young tomato fruit library (cv VFNT Cherry LA1221, as described by Narita and Gruissem [1989]), using a random primer labeled (Prime-a-Gene) PFP-specific probe, as described in the previous section. Plasmid DNA was prepared by in vivo excision, according to the manufacturer's instructions (Stratagene).

PCR Methods and Sequence Analysis

PCR reactions were performed in 25-μL volumes, using Promega Taq DNA polymerase in 10 mM Tris-HCl, pH 9.0, 50 mM KC1, 0.1% Triton X-100, 0.1 mM each of dATP, dGTP, dCTP, and dTTP, and 2 mM MgCl2, with primers at 1 μM each. PCR reactions were performed in thin-walled 0.5-μL tubes in a thermal cycler (model PTC-200; MJ Research, Watertown, MA) for one cycle of 94°C for 2 min, followed by 25 to 30 cycles of 94°C for 30 sec, 54°C to 60°C for 30 sec, and 72°C for 2 min.

Reverse transcriptase-PCR (RT-PCR) was performed as follows. Total RNA in reverse transcription buffer (Promega) was treated with RNase-free DNase at 37°C for 10 min and then heated at 90°C for 10 min. Reverse transcription was primed with oligo(dt)15 by using the Moloney murine leukemia virus RT enzyme (Promega) at 42°C for 2 hr. Reverse transcription was stopped by heating at 95°C for 5 min. After reverse transcription, PCR was performed using an LeT6-specific primer (NST1, 5'-AGATGATCCATTTCTATCCA-3') and a PFP-specific primer (PFP1, 5'-AGGTGGAACAGCACTGC-3'), using one cycle of 94°C for 1 min followed by 36 cycles of 94°C for 30 sec, 54°C for 1 min, and 72°C for 1 min. After PCR, the product was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

The junction region in the fusion cDNAs was amplified from Me genomic DNA by using an LeT6-specific primer (CNST12, 5'-AAGGAAAGAAAAGAGTGTG-3') and a PFP-specific primer (PFP1). The PCR product was gel purified using a Qiagen gel extraction kit and cloned using the Promega pGEM-T vector system.

The region of PFP that spans the region of gene fusion in Me was amplified from both cultivar Rutgers and Me genomic DNA by using primer PFP1 (which is located in exon 13) and a primer in exon 15 (PFP5, 5'-CATTCTTCTCCTTGGAAAGC-3'), which was designed from a tomato PFP cDNA clone. The PCR products were gel purified using a Qiagen gel extraction kit and cloned using the Promega pGEM-T vector system.

Nucleotide sequence determination was performed at the University of California, Davis, Division of Biological Sciences automated DNA sequencing facility, using dye terminator chemistry on an ABI Prism 377 DNA sequencer (Perkin-Elmer, Norwalk, CT). Sequence was analyzed using the SEQUED program and compared with database using the BLAST program (Altschul et al., 1990).

Mapping Methods

The PFP gene was mapped to a specific chromosome location by using F2 populations of a cross between Lycopersicon esculentum and L. pennellii (Bernatzsky and Tanksley, 1986; Paterson et al., 1988; Tanksley et al., 1989). A region spanning introns 13 and 14 of PFP was amplified using PCR (PFP1, 5'-AGGTGGAACAGCACTGC-3'; and PFP5, 5'-CATTCTTCTCCTTGGAAAGC-3'), and the resulting DNA fragment (350 bp) was purified, digested with TaqI, and electrophoresed on a 2% agarose gel. Each F2 individual was scored for parental type at the PFP locus, and the data were analyzed using the MapMaker-QTL (Paterson et al., 1988) program to determine map location.

RNA in Situ Localizations

Slides for RNA in situ hybridization used 35S-labeled riboprobes (Meyerowitz, 1987) and digoxigenin-labeled riboprobes (Coen et al., 1990) with some modifications. After we performed anti-digoxigenin antibody labeling and washes, slides were left for the duration of overnight to 2 days in wash buffer A (Coen et al., 1990) at 4°C before we proceeded to the detection steps. Sections were dehydrated through a graded ethanol series, cleared in Histoclear (National Diagnostics), and mounted in Permount. Under these conditions, hybridization temperatures were approximately at or 8°C below the Tm, whereas the washes were at 12°C above Tm, calculations were performed according to Sambrook et al. (1989)). These hybridization and wash conditions were more stringent than those used for RNA gel blot analysis.

Transgenic Methods

The LeT6 cDNA was cloned between the double cauliflower mosaic virus (CaMV) 35S 5' region and the polyadenylation region from the Tml gene in the vector pCGN187 (Comai et al., 1990). This chimera gene was then cloned into the binary vector pCGN1549 (McBride and Summerfelt, 1990). The 35S-LeT6 binary vector was then transformed into Agrobacterium tumefaciens LBA4404 (Hoeckema et al., 1983) by using the freeze-thaw method (An et al., 1988). Tomato cotyledons (cv NC8276) were transformed, and transgenic plants were regenerated as described by Fillatt et al. (1987).

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