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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).

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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 *stm1* and soybean *sbh1* genes, whereas *Tkn1* might be most similar to Arabidopsis *knat1* and slightly less similar to maize *rs1* and *kn1* (G. Bharathan, B.-J. Janssen, E. Kellogg, and N. Sinha, submitted manuscript). Thus, results obtained from the overexpression of *kn1* in tomato are unlikely to be identical to those obtained from overexpression of *LeT6*, or even *Tkn1*, in tomato.

Although sequence similarity between the coding regions of genes important in morphogenesis reveals the relatedness of organisms in which they occur (Kappen et al., 1993; Munster et al., 1997), there is little information about how variation in these genes may have led to altered forms in related species. Alterations in patterns of gene expression can cause dramatic changes in morphology and may have been important in driving morphological evolution (Doebley et al., 1997). However, morphologically important genes may also have been altered by exon-shuffling events. The discovery of the modularity of proteins involved in signal transduction cascades (Bent, 1996) indicates that exon shuffling may have played an important role in the evolution of signaling molecules (Gilbert, 1978; M. Long et al., 1996). Exon shuffling caused by gene fusions in signaling molecules has been implicated in a number of human cancers (Shtivelman et al., 1985; Mellentin et al., 1989; Glassman, 1995). These fusion events typically result in change in function of one or both of the donor genes and may also result in novel expression patterns of the genes in question by bringing the two partners in the fusion event under the control of a promoter coming from one of the two genes.

We have compared spontaneous overexpression and CaMV 35S–regulated overexpression of an endogenous homeobox gene, *LeT6*, by combining analysis of a naturally occurring leaf mutation in tomato with a transgenic approach. Our results indicate that a spontaneous gene fusion led to overexpression of a novel *LeT6*-related transcript in the *Mouse ears* (*Me*) mutation in tomato. The leaf phenotypes seen in *Me* closely approximate those seen when *LeT6* is overexpressed under control of the CaMV 35S promoter. Both the level and pattern of *LeT6* expression are altered in the *Me* mutation. The phenotypes are not regulated merely by an increase in the level of the LeT6 homeodomain protein. Rather, the domain of expression of the fusion protein is altered, leading to phenotypes distinctly different from those of the wild type. The fusion event appears to be an exon capture phenomenon, and it allows us to envision how genes may acquire altered regulation and gain or lose exons. Such genomic alterations may account for rapid phenotypic changes between closely related groups.

RESULTS

LeT6 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

primordium is seen on leaf 4 of the *Me* plant. In addition, whereas leaf 2 in the wild type shows the differentiation of a trichome (Figures 1H and 1I, arrows) at its tip, indicating its basipetal differentiation pattern, leaf 2 on the *Me* apex shows trichomes in the midregion of the primordium. Such alterations in leaf maturation (indicated by presence of mature trichomes) are seen in all *Me* apices examined. The primordium for leaf 3 is narrower in the *Me* plant than in the wild type. Thus, the patterns of leaflet initiation, basipetal leaf maturation, and leaf shape are all altered relative to the wild type in the *Me* mutation.

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 blots 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–*kn1* 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 *kn1*, and may be an indication that just like *kn1*, 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 *kn1* 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 P_0, 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 P_0 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

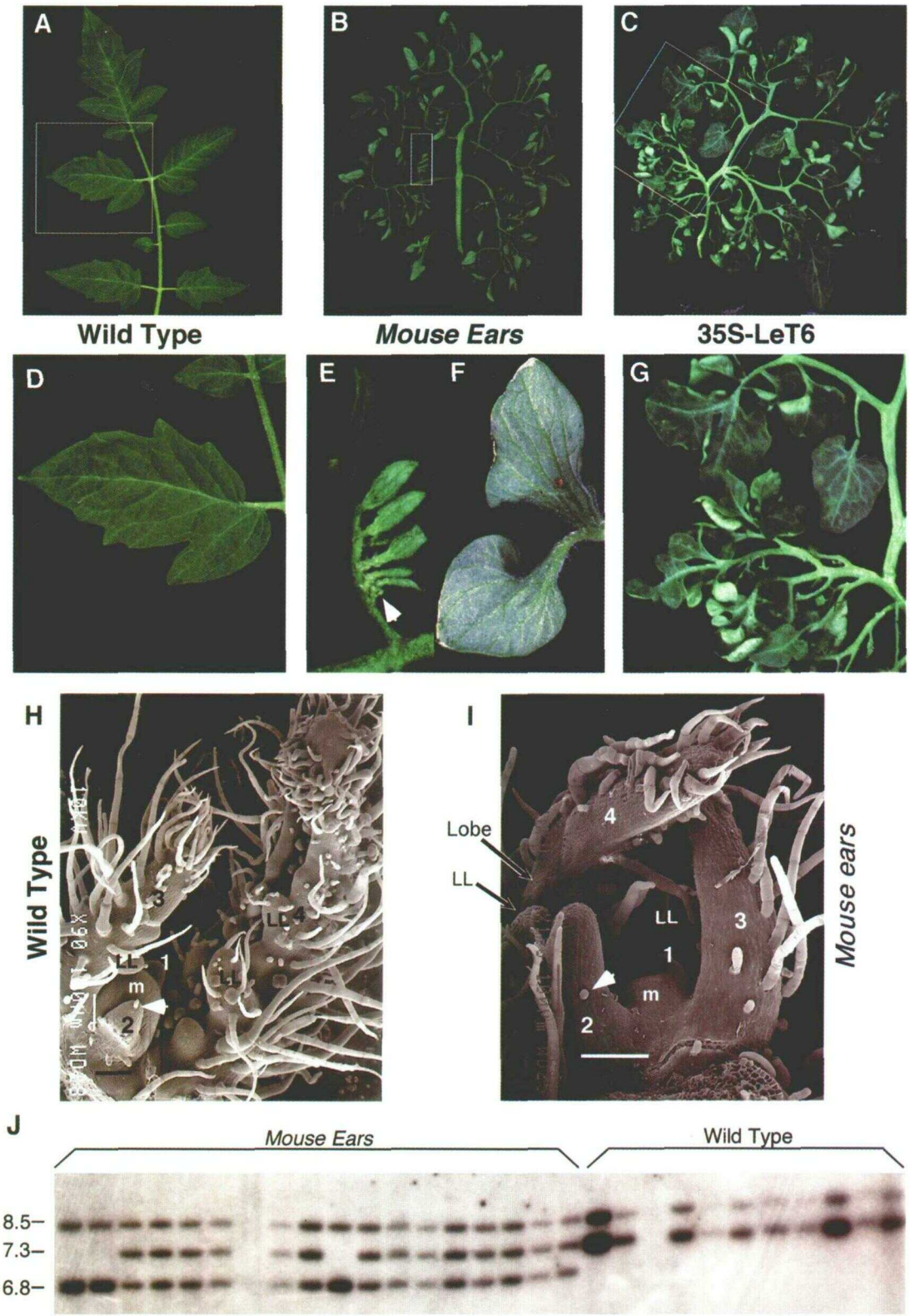

Figure 1. Phenotypic Comparison of Wild-Type, *Me*, and 35S–*LeT6* Plants.

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.

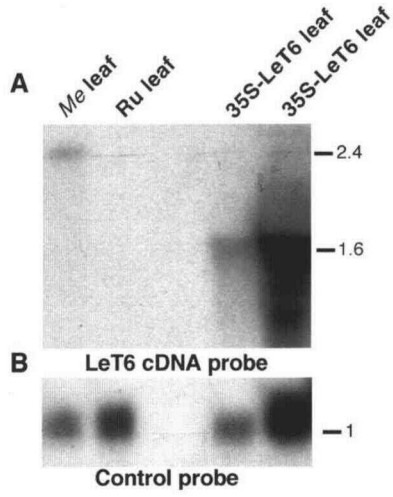

Figure 2. Gel Blot Analysis of RNA from Mature Leaves of Wild-Type (cv Rutgers), *Me*, and 35S–*LeT6* Plants.

(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.

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.

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

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 palmate 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.

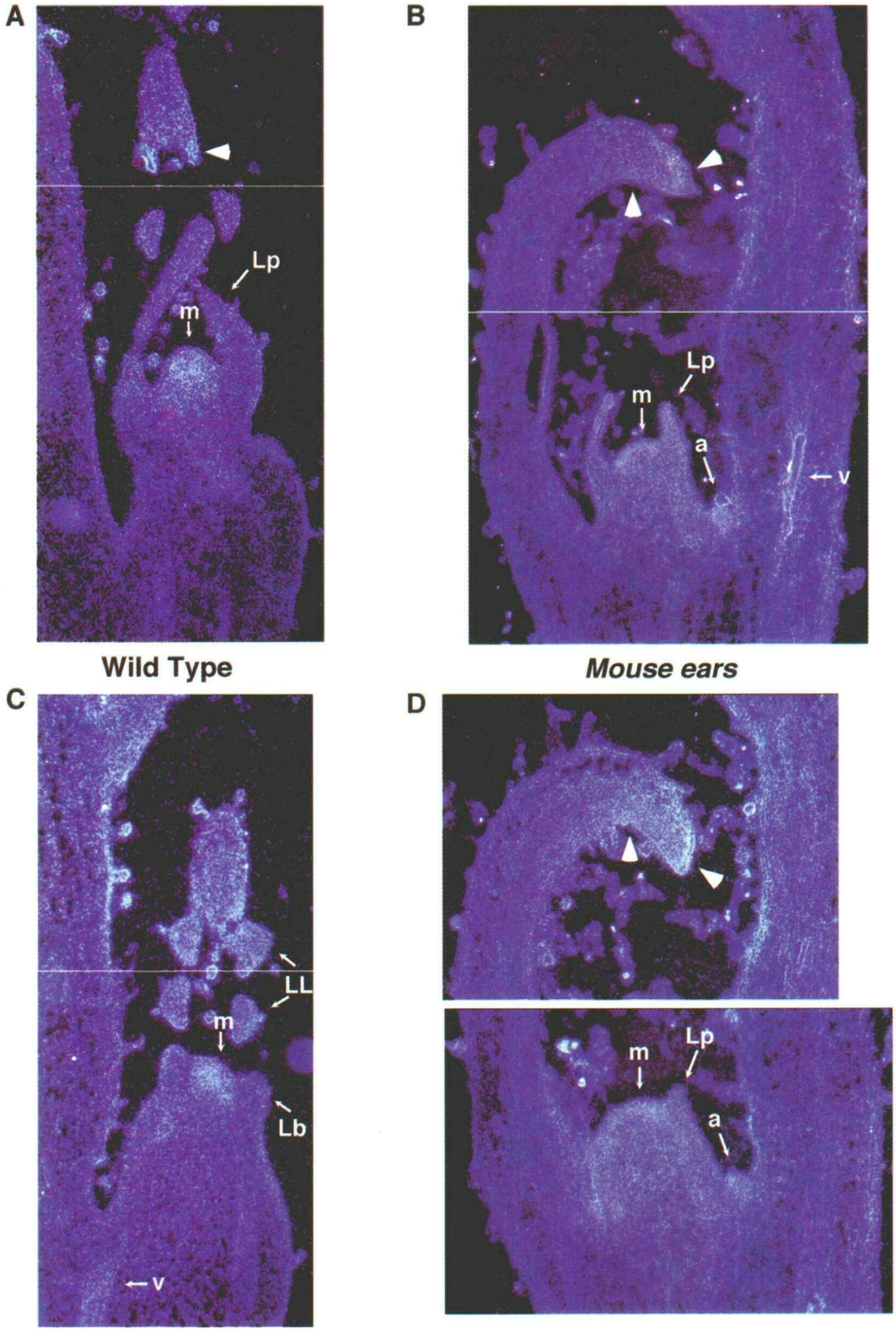

Figure 3. In Situ Localization of the *LeT6* Transcript in Wild-Type and *Me* Shoot Apices.

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 PFP end of the fusion was identical in both classes, but the *LeT6* ends were different. In the first class of cDNAs (PFP–LeT6–1), 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 other two 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).

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.

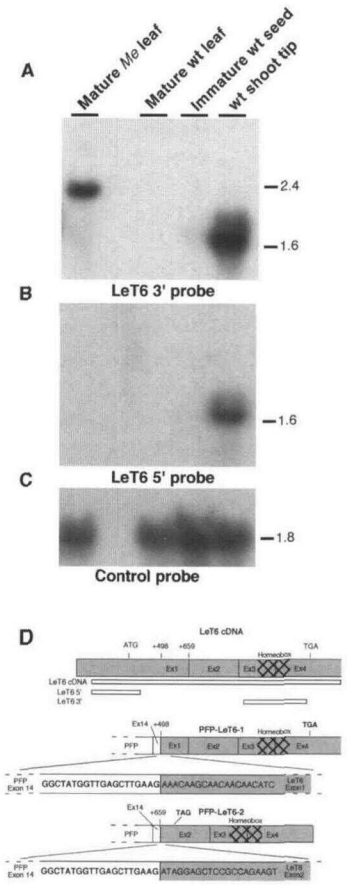

Figure 4. RNA Gel Blot Analysis of Various Tissue Samples from Wild-Type (cv Rutgers) and *Me* Plants.

(A) Total RNA was fractionated by gel electrophoresis, blotted onto a nylon membrane, and hybridized with an *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 *LeT6* 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 *LeT6* 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

Figure 5D. 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 35S–*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 addition to a fused PFP transcript in *Me* plants, further confirms that in *Me*, the PFP locus is duplicated.

We mapped the chromosomal location of PFP by using the F_2 populations of a cross between *L. esculentum* and *L. pennellii* (Bernatzsky and Tanksley, 1986; Paterson et al., 1988; Tanksley et al., 1989). We were able to unambiguously place PFP on chromosome 2, near marker TG454 (lod score, 9.78; confidence limit, 99%). TG454 is ~8.4 cM distal from Prx-2,3. This is also the genetic map distance between Prx-2,3 and *Me*. In addition, *LeT6* maps coincident with PFP on chromosome 2. This indicates that *Me*, PFP, and *LeT6* are very close to each other on chromosome 2, and there is most likely a tandem duplication of the PFP locus in the *Me* mutation.

DISCUSSION

The cultivated tomato has a unipinnately compound leaf. However, considerable variation in leaf morphology is seen in other members of the tomato family. Sometimes this variation occurs in members of the same genus, indicating that leaf form is very plastic and easily altered in the family. We have shown that spontaneously arisen and engineered alterations in levels and domains of expression of a homeobox gene can cause dramatic changes in leaf morphology. In the *Me* mutation, a gene fusion between the PFP β subunit and a homeobox gene *LeT6* leads to a fusion product that has both increased levels and altered patterns of gene expression, with consequences for leaf morphology in the mutation.

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 35S–*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

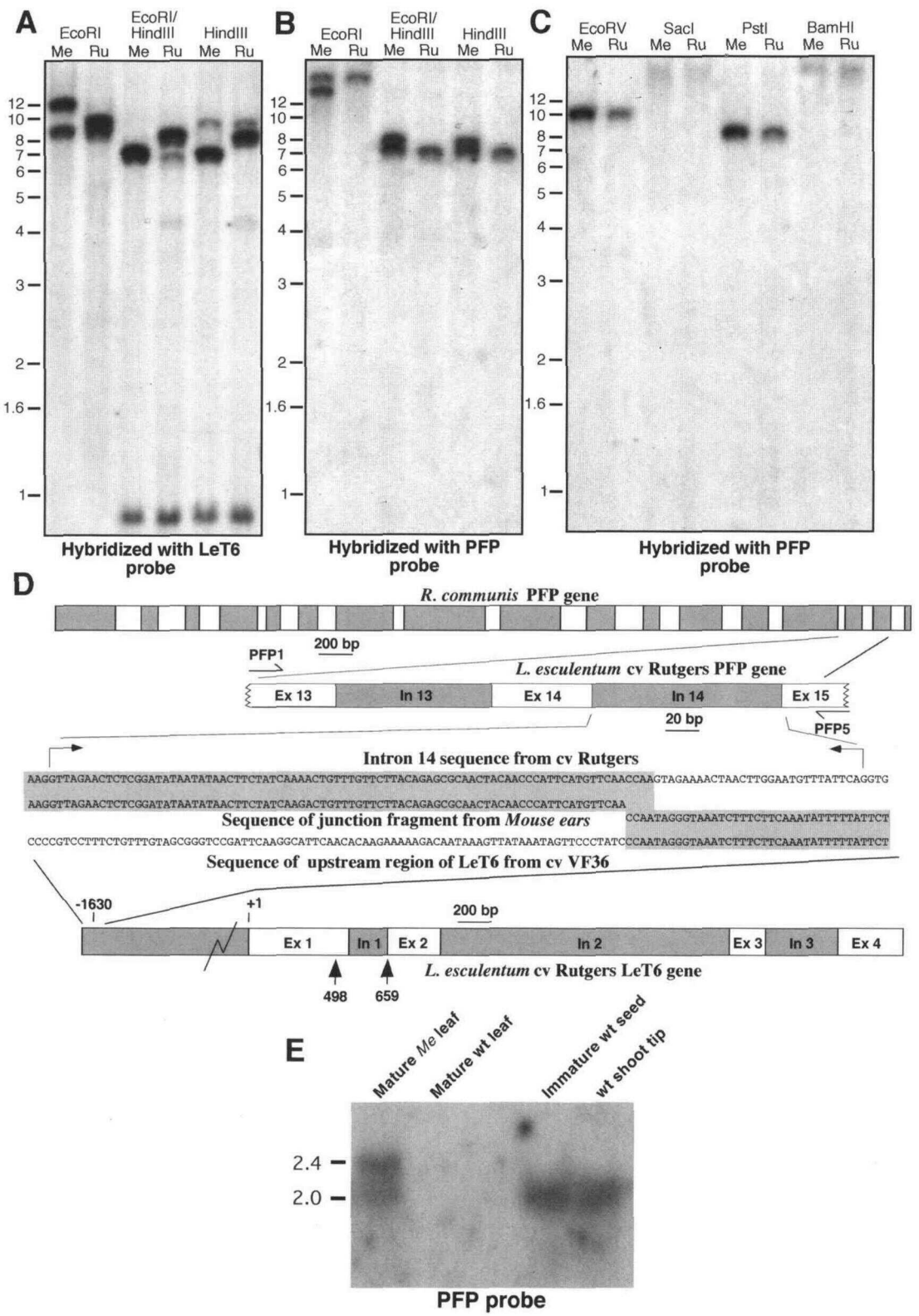

Figure 5. DNA Blot Analysis of the PFP and *LeT6* Loci.

and Lam, 1996). Recent domain-swapping experiments for KNOX proteins indicate that functional specificity resides in the ELK and homeodomain regions (Serikawa 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 (Benfey 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

Figure 5. (continued).

(A) to **(C)** DNA was prepared from *Me* and *L. esculentum* cv Rutgers (Ru) tissue, restricted with endonucleases EcoRI, EcoRI and HindIII, and HindIII alone, electrophoresed on a 1% agarose gel, and blotted to a nylon membrane. The membrane was hybridized first with an *LeT6* cDNA probe **(A)** and then stripped and rehybridized to the PFP β–specific portion of the *Me* cDNA clone **(B)**. DNA from *Me* and cultivar Rutgers tissue was restricted with endonucleases EcoRV, SacI, PstI, and BamHI, electrophoresed on a 1% agarose gel, and blotted and hybridized to the PFP β–specific portion of the *Me* cDNA clone **(C)**. Identical sizes of hybridizing fragments and intensity of the hybridizing fragments indicate that the PFP β–hybridizing segment of DNA is duplicated in *Me* and identical for >10 kb with native PFP β sequences. Lengths at left are in kilobases.

(D) Shown is a diagram of the gene fusion in *Me*. The structure of the *R. communis* PFP β gene with the introns shaded is shown for reference. PCR primers were designed to a *L. esculentum* cv VFNT Cherry PFP β cDNA clone, and the region from exon 13 (PFP1) to exon 15 (PFP5) was amplified from cv Rutgers DNA, cloned, and sequenced. PCR was also used to amplify the *Me* genomic region covering the fusion using primer PFP1 and a primer within the *LeT6* coding region. This region was also cloned and sequenced. A partial genomic clone of *LeT6* from cultivar VF36 was sequenced over the region corresponding to the fusion in *Me*. An alignment of the sequences at the fusion region is shown with regions of sequence homology indicated by shading. Arrows indicate the splice sites utilized to generate the two PFP–LeT6 fusion RNAs. Bent arrows indicate the boundaries of PFP intron 14. The jagged line indicates that the upstream region of LeT6 is not drawn to scale. Ex, exon; In, intron.

(E) RNA gel blot (the same blot as shown in Figures 4A to 4C) was probed with a PFP β–specific DNA fragment 5′ of the fusion point. The position of the upper band in mature *Me* leaf was the same as that of the band in mature *Me* leaf in Figure 4A. Markers at left are given in kilobases. wt, wild type.

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_0 primordium 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_0 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_0 region of simple-leaved tobacco.

The Arabidopsis *stm1* 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_0 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 tomato. Downregulation in P_0 developing 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 tomato, and the expression patterns of a *kn1*-like gene from tomato (*Tkn1*) have been described in floral meristems and leaf primordia (Hareven et al., 1996). In this study also, no downregulation in P_0 was observed. Either tomato shoot apices and leaf primordia are like those in maize and Arabidopsis, in which case the P_0 downregulation of *knox* genes in maize and Arabidopsis is coincidental and inconsequential to leaf initiation and development, or tomato shoot apices and leaf primordia are very different from those in maize and Arabidopsis, and downregulation of *knox* genes in P_0 is not a prerequisite for leaf initiation and development in tomato. The one striking difference between these two sets of organisms in seen in their leaves, with those in maize and Arabidopsis being simple and those in tomato being compound. Shape changes are rarely seen in floral organs of tobacco or Arabidopsis plants overexpressing the class 1 *knox* genes. Expression in the P_0 stage of floral organ primordia in tomato may have no consequences for shape and complexity, because these organs may be more rigid in their developmental potential. We speculate that expression in the P_0 leaf primordium leads to compounding in tomato leaves. This may be due to greater developmental plasticity in leaves than in floral organs. The nature of compound leaves has been debated thoroughly (Eames, 1961; Hickey and Wolfe, 1975; Hagemann, 1984; Merrill, 1986). They have been considered to be either simple leaves that proliferate extra segments or structures that have partially indeterminate features. Because the class 1 *knox* genes are expressed in the shoot apical meristem and regions of the stem, expression of this class of genes in the compound leaf of tomato suggests that the tomato compound leaf has some indeterminate features.

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 proto-oncogene (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 (Glassman, 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 cm^2 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 on a slide warmer. Slides were deparaffinized 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 CO_2. 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 election 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′-AAATGGTTAGCAAGAACATT-3′), as was the 5′ specific probe (primer NST2, 5′-TTTTTTTCAGTGTGTGTGTGA-3′; primer JT15, 5′-CTGCATTGTTATTGTTAGTT-3′). After hybridization, filters were washed twice in 0.5 × SSPE (SSPE made according to Sambrook et al. [1989]), 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 [T_m] 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 (Pruitt 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-mercaptoethanol. SDS (1 mL of a 20% solution) was added, and the mixture was incubated at 65°C for 10 min. Prechilled 5 M potassium acetate (10 mL) was added, and the samples were mixed, incubated on ice for 20 min, and then centrifuged at 16,000*g* 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 16,000*g* 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 reprecipitation.

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 λOCUS vector, using a directional cDNA library construction system (Novagen, Madison, WI). The primary library had a titer of $\sim 10^6$ 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′-AAATGGTTAGCAAGAACATT-3′; and JT6, 5′-TCATCGACCCCCAGGCTG-3′). Plasmid DNA was prepared using 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 KCl, 0.1% Triton X-100, 0.1 mM each of dATP, dGTP, dCTP, and dTTP, and 3 mM $MgCl_2$, with primers at 1 μM each. PCR reactions were performed in thin-walled 0.5-mL 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 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 (NST11, 5′-AGATGATCCATTTCTATCCA-3′) and a PFP-specific primer (PFP1, 5′-AGGTGGAACAGCACTCACTGC-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′-AAGGAAGAAAAAGGATGTTG-3′) and a PFP-specific primer (PFP1). The PCR product was gel purified using a Qiagen (Chatsworth, CA) 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′-CATTCTTCCCTCTTTGAAGC-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 SEQED program and compared with databases using the BLAST program (Altschul et al., 1990).

Mapping Methods

The PFP gene was mapped to a specific chromosome location by using F_2 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′-AGGTGGAACAGCACTCACTGC-3′; and PFP5, 5′-CATTCTTCCCTCTTTGAAGC-3′), and the resulting DNA fragment (350 bp) was purified, digested with TaqI, and electrophoresed on a 2% agarose gel. Each F_2 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 ^{35}S-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 T_m, whereas the washes were at 12°C above T_m (T_m 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 pCGN2187 (Comai et al., 1990). This chimeric 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 (Hoekema 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 Fillatti et al. (1987).

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