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The Making of a Compound Leaf: Genetic Manipulation of Leaf Architecture in Tomato

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Summary

The most distinctive morphogenetic feature of leaves is their being either simple or compound. To study the basis for this dichotomy, we have exploited the maize homeobox-containing *Knotted-1* (*Kn1*) gene in conjunction with mutations that alter the tomato compound leaf. We show that misexpression of *Kn1* confers different phenotypes on simple and compound leaves. Up to 2000 leaflets, organized in compound reiterated units, are formed in tomato leaves expressing *Kn1*. In contrast, *Kn1* induces leaf malformations but fails to elicit leaf ramification in plants with inherent simple leaves such as Arabidopsis or in tomato mutant plants with simple leaves. Moreover, the tomato *Kn1* ortholog, unlike that of Arabidopsis, is expressed in the leaf primordia. Presumably, the two alternative leaf forms are conditioned by different developmental programs in the primary appendage that is common to all types of leaves.

Introduction

All types of leaves, regardless of their eventual architecture, arise as dorsiventral appendages from the flanks of the shoot apical meristem. The dorsiventral nature of leaf primordia contrasts with axillary branches or stems that arise as radial primordia (Cutter, 1971; Kaplan, 1973). In angiosperms, vegetative leaves come mainly in two basic arrangements: simple and compound. The single blade of the simple leaf, as well as the independent blades (leaflets) of the compound leaf, can be sessile or can be carried on a petiole, and their margins can be entire, lobed, parted, dentate, or palmate. Leaflets of a compound leaf are distinguished from the leaves, as only the latter form axillary buds (see Smith and Hake, 1992).

Variations in leaf shape have been analyzed by genetic or anatomical means in maize, pea, cotton, and a wide variety of other species (Marks, 1987; Dolan and Poethig, 1991; Sinha et al., 1993a; Steeves and Sussex, 1989), and growth parameters of blade units in simple leaves have been studied by clonal analysis (Poethig, 1987; Poethig and Sussex, 1985; also Freeling, 1992; Smith and Hake 1992). However, at present, no coherent developmental-genetic framework for leaf morphogenesis has been derived, and the pathways that determine a simple versus compound leaf have not been investigated. Compound leaves have been considered to represent the reiteration of the program that makes simple leaves. To study the molecular-genetic basis for this dichotomy between simple and compound leaves, we have exploited the maize homeobox-containing *Knotted-1* (*Kn1*) gene in conjunction with a range of mutations that alter the tomato compound leaf.

The diverse forms of plant organs are shaped by developmental events in their respective meristems (Sussex, 1989), and the identification of *Kn1* as a meristematic homeobox gene has permitted molecular-genetic analyses of leaf morphogenesis. Dominant mutations in the *Kn1* locus of maize result in distinct alterations in cells along the vasculature of the blade. Formations of pocketed outgrowths (knots) on lateral veins, overall growth retardation, wider leaves, distorted patterns of lateral veins, disappearance of the ligule appendage, and ectopic formation of sheath tissues on blades characterize the KNOTTED syndrome (Freeling and Hake, 1985; Sinha and Hake, 1994). The *Kn1* gene was cloned by transposon tagging and shown to represent a conserved class of plant genes coding for homeodomain-containing plant proteins (Hake et al., 1989; Volbrecht et al., 1991; Hake, 1992; Kerstetter et al., 1994; Becraft and Freeling, 1994). It was convincingly shown that the dominant nature of *Kn1* mutations is a consequence of its ectopic expression in the lateral veins of the leaf blade (Smith et al., 1992; Jackson et al., 1994). The *Kn1* gene is normally expressed in vegetative and inflorescence apical meristems, but not in leaf primordia, nor in developing leaves or in floral organs. When the *Kn1* gene was misexpressed in dicot plant species with simple leaves, like tobacco and Arabidopsis, severe morphogenetic alterations were induced (Sinha et al., 1993b; Lincoln et al., 1994). The leaves of these species turn lobed and rumpled, as well as shorter and wider and, in extreme cases, ectopic shoots appear on the main veins. Growth retardation and loss of apical dominance have also been observed. Significantly, and despite the excessive meristematic activities causing all the growth malformations, leaves of Arabidopsis and tobacco plant overexpressing *Kn1*, like those of maize *Kn1* overproducers, remain simple.

We hypothesize that simple and compound leaves are the result of different patterns of meristematic activities. We also propose that simple leaves are morphogenetically rigid, while compound leaves are developmentally more flexible, thus permitting the phenotypic manifestation of a wide scope of gene mutations. Tomato is well suited for the analysis of simple versus compound in isolation from other parameters of leaf shape, since a range of dominant and recessive mutations, which change leaves from supercompound to simple, are available (Stevens and Rick, 1986; Figure 3) and since all appendages of its compound leaf are identical.

We suspected that the morphogenetic versatility of the tomato compound leaf, in conjunction with the demonstrated meristematic function of the *Kn1* gene family

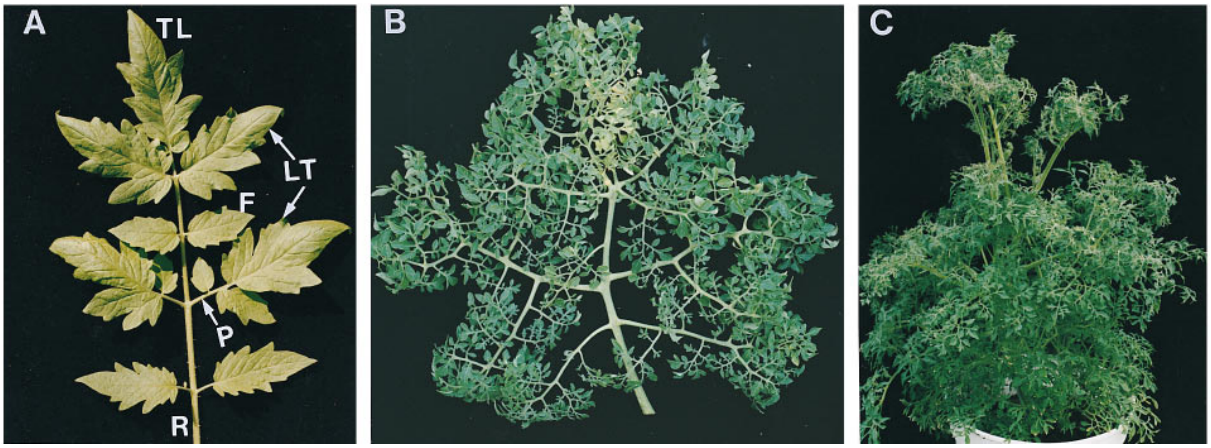

Figure 1. Ultracompound Leaves in Kn1-Expressing Tomato Plants

(A) Wild-type compound leaf: the prototype unit. The terminal leaflet (TL) emerges first and the pairs of lateral leaflets (LT) appear in basipetal order. Note that all leaflets are anatomically similar, each is petiolated (P), and with serrate margins. Folioles (F) appear occasionally between leaflets along the rachis (R) or on either side of the petioles.

(B) A supercompound leaf of transgenic tomato plants expressing the *Kn1* gene. Terminal and lateral leaflets of the prototype unit now acquire the compoundness of the wild-type leaf.

(C) Transgenic tomato plant expressing *Kn1* under the control of the *35S* promoter. Note the bushy appearance of the plant, the consequence of lost apical dominance.

in both mono- and dicotyledonous plants, would provide new opportunities to study leaf arrangement. In this report, we show that the ubiquitous misexpression of the maize *Kn1* gene in the tomato leaf confers dramatic additional orders of subdivisions on the already compound leaf. Such a ramification is completely prevented in the simple leaves of the tomato mutant *Lanceolate* (*La*). Our observations suggest that a compound leaf is not a trivial reiteration of a simple leaf and that the making of either leaf type depends on mutually exclusive growth patterns.

Results

Misexpression of *Kn1* Makes the Tomato Leaf Strikingly More Compound

In plants of the wild-type progenitor line, the prototype compound leaf is composed of a midvein (rachis), a terminal (distal) leaflet, and three to four pairs of lateral, petiolated, and dentate leaflets. Occasionally, several additional folioles emerge on the midvein between leaflets or on the petioles of the lateral leaflets (Figure 1A). The effect of the *Kn1* gene on morphogenesis of compound leaves was observed by generating transgenic tomato plants. Generated in the determinate TRK9/8 and VF36 lines were 42 kanamycin-resistant primary transformants (T1 plants) expressing *Kn1* under the control of the potent and ubiquitous cauliflower mosaic virus *35S* promoter (Benfey and Chua, 1990). All but five transformants exhibited extreme alterations in the degree of structural ramification of the leaf. In the 37 independent M series T1 primary transformants displaying altered morphogenesis, mature leaves are subdivided to the fourth, fifth, or sixth order, forming a supercompound leaf (Figure 1B). The appearance of supercompound leaves is always associated with growth retardation and the loss of apical dominance, resulting in dwarfed, bushy plants (Figure 1C).

Ramified primary, secondary, and tertiary lateral leaflets excised from the leaf shown in Figure 1B are illustrated in Figure 2A. In such leaves, each lateral extension acquires the complexity of the primary compound design, as shown in Figure 1A. Independent transformants yield different types of ultracompound leaves, producing leaflets with altered vein-to-lamina ratio. The type of leaflets shown in Figures 1B and 2B are the most prominent. Reiteration of such units (the term phyllomere is suggested to describe a given prototypic leaf architecture) results in an increase in the number of leaflets from 9 to 700–2000. However, note that although the overall number of leaflets per compound leaf is greatly increased, the dimensions of the leaf remain mostly unchanged. The excessive proliferation of lateral leaf appendages that is associated with alterations of the lamina-to-vein ratio in a given leaflet is also associated with altered relative size of the terminal leaflets in each compound unit, or with the modified spacing of such units along the midveins. Leaves representing extreme alterations of these parameters, which were formed by independent transformants, are shown in Figure 2C.

Several additional major anatomical features characterize *Kn1*-expressing primordia and leaf blades in *35S::Kn1* plants. Nearly equal adaxial (toward the center) and abaxial cell growth confers, from the outset, an erect shape on the wild-type leaf primordium. First and second primordia of the lateral leaflets appear in a basipetal order when the primary, peg-like, primordium of the wild-type leaf reaches about 300–400 μm and 800 μm in length, respectively (Dengler, 1984; stars in Figure 2D). In *35S::Kn1* plants, multiple lateral leaflet buds develop prematurely, in a much more distal position on the leaf primordium (stars in Figures 2E and 2F). New meristematic centers in a secondary leaflet primordium show

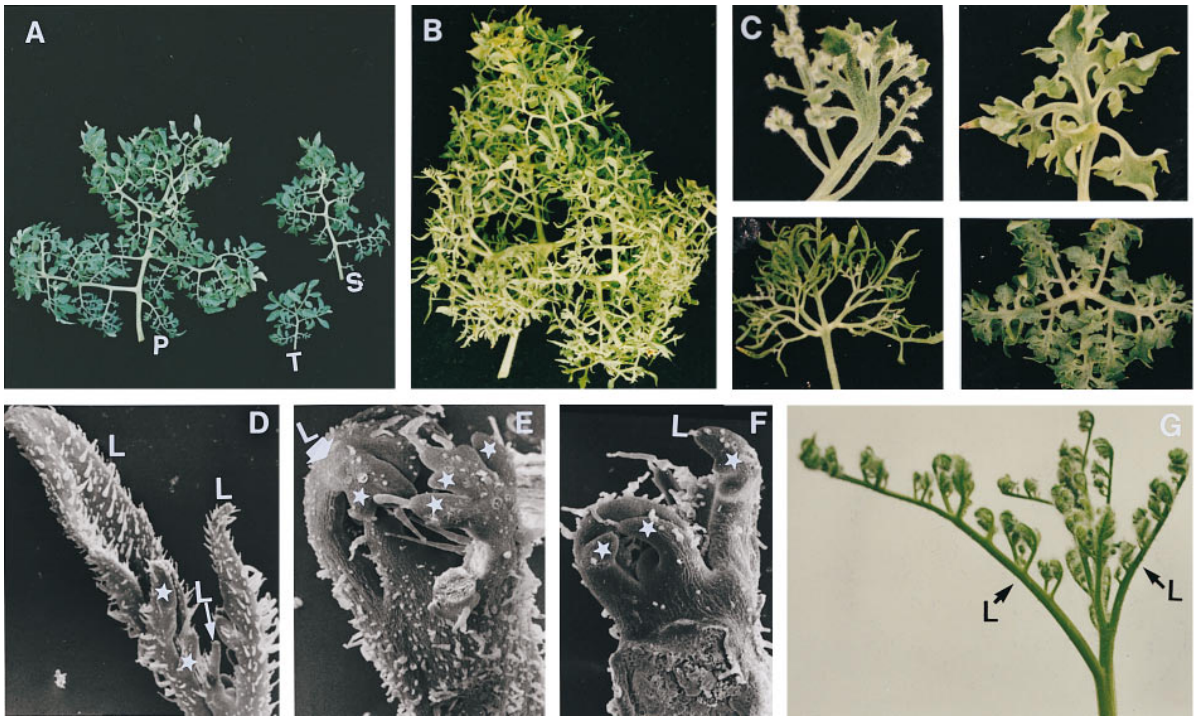

Figure 2. Growth Parameters in *Kn1*-Expressing Tomato Plants

(A) Degree of subdivision of a single supercompound leaf. Primary (P), secondary (S), and tertiary (T) lateral leaflets excised from the leaf shown in Figure 1B illustrate the ramification of the supercompound leaf.

(B) A supercompound leaf with altered lamina-to-vein ratio. This type of leaf and the one shown in Figure 1B are the most prominent among tomato plants expressing the *35S::Kn1* transgene.

(C) Extreme variation in blade and leaflet morphology in tertiary branches of supercompound leaves of four independent primary transformants.

(D–E) Scanning electron micrographs (SEMs) of wild-type and *35S::Kn1* transgenic apices. (D) Wild-type apex with leaf primordia. Two older leaf primordia (L) and a newly emerging one (marked with an arrow) are seen. Leaflet primordia are marked with stars. Note the erect stage of primordia and the relative sites of emerging lateral leaflets.

(E–F) Apices of *35S::Kn1* plants with premature appearance of distal lateral appendages (stars) and inward curving of the primordia.

(G) Fiddle-head shape of supercompound leaves (L) before expansion.

a similar pattern of leaflet proliferation. Leaf primordia in the shoot apex, as well as young leaflets of the emerging leaf of *Kn1*-expressing plants, presumably as a consequence of unequal abaxial and adaxial growth, tend to curl inward toward the center and display a "fiddle-head" shape, reminiscent of fern leaves. This shape is retained until just prior to final leaf expansion (Figure 2G).

In both tomato and tobacco leaves expressing the *35S::Kn1* transgene, the prominence of the midvein is reduced to a condensed palmate-like design. The tertiary vein system is more diffused, and areoles (the smallest lamina fields confined by veins) are 2- to 3-fold larger. Every vein thus serves more cells (data not shown).

Unlike leaves, morphology of the inflorescences, flowers, and floral organs of tomato are not visibly affected by the misexpression of the *Kn1* gene. Two thirds of the primary transformants are fertile, and the *35S::Kn1* phenotype is transmitted to T2 plants in the expected Mendelian proportions for a single locus–dominant mutation. The early degeneration of flowers in the nonfertile one third of the transgenic plants is attributed to secondary effects such as overall growth retardation.

Analysis of *Kn1* mRNA in transgenic plants (shown later as part of Figure 4) revealed high levels of expression in leaves and flowers of all affected plants, irrespective of the severeness of the leaflet phenotype or the degree of subdivision (Figure 4A, lanes 1–4 and 9–12).

Kn1 Induces Leaf Ramification in the Compound Leaves of *Petroselinum*, but Not in the Simple Leaves of *La* Plants

Phenotypic consequences of misexpression of *Kn1* in the simple leaves of maize (Sinha and Hake, 1994) or in transgenic rice, tobacco, and Arabidopsis plants are restricted to local distortions of the blade (Matsuoka et al., 1993; Sinha et al., 1993; Lincoln et al., 1994). In contrast with the dramatic effect on tomato leaves, the misexpression of *Kn1* in these other species does not change the basic simple design of the leaves. The difference could be attributed to unknown species-specific factors, or to inherent variations in the programs that condition simple and compound leaves. To address this question directly in a single plant species, misexpression of *Kn1* was examined in several mutants of tomato.

Petroselinum (Pts) and *La* represent the two extreme variations of the compound leaf. *Pts* leaves have elaborate leaflets with three to four pairs of secondary leaflets

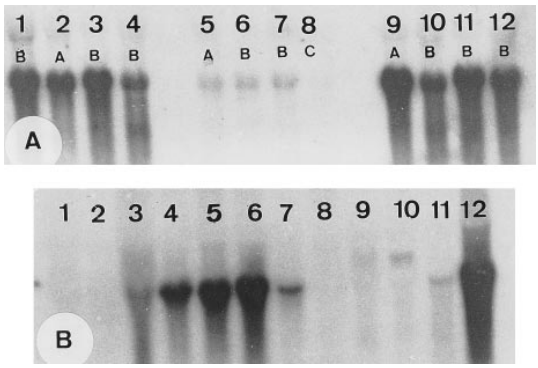

Figure 4. Blot Analysis of *Kn1* and *TKn1* Transcripts in Total RNA Samples

(A) Expression of *Kn1* in floral buds of independent primary (T1) transformants driven by the 35S and dUTPase promoters: lanes 1–4 and 9–12; *35S::Kn1* transgenic plants; lanes 5–8; *PdUTPase::Kn1* transgenic plants.

The letters a, b and c, which appear above lanes 1–12, denote strong, medium, and weak phenotypic expression, respectively.

(B) Expression pattern of the tomato TKn1 gene in different plant organs: 3 cm long leaves of the 93-137 wild-type line (lane 1); 5 cm long leaves of 93-137 plants (lane 2); 3–5 mm long leaves of 93-137 plants (lane 3); 5 mm long intact apices of 93-137 plants (lane 4); 10 mm long stem sections of 93-137 plants (lane 5); 5 mm long stem sections of 93-137 plants (lane 6). Apices include the actual apex, up to five-leaf primordia, the longer of which is 5 mm, and two to four floral primordia of the stage shown in Figure 6. Stem sections are barren stems 5 mm long just below the shoot apex.

Mature flowers two to three days before anthesis (lane 7). Floral organs of mature flowers: sepals (lane 8); petals (lane 9); stamens (lane 10); carpels (lane 11); The amount of carpel RNA loaded is only one half of that loaded for the other organs; *Anantha* floral meristems (lane 12). Flowers are good representatives of the level of *Kn1* transcripts because they exhibit no phenotypic variation between and within transgenic plants.

(Figure 3A), each of which resembles the wild-type phyllomere (compare with Figure 1A). Such leaves are said to be divided to the third order. A simple and entire leaf, composed of one petiolated blade similar to that of wild-type tobacco or Arabidopsis, is formed in plants heterozygote to the dominant *La* gene (*La*/+) (Figure 3E). Homozygote *La/La* seedlings are practically lethal, as no apical shoot meristems are produced (Mathan and Jenkins, 1962; Stettler, 1964; Caruso, 1968). Three mutants in which compoundness is intermediate between wild type and *La* illustrate the genetic control of the development of the basic prototype. In *entire (e/e)* homozygote plants, a pair of reduced and sessile lateral leaflets is fused to the terminal one to generate a pseudosimple leaf (Figure 3B). The recessive *potato-leaf* gene (*c/c*, sometimes referred to as *solanifolia*) permits the formation of only two, rather than three to four pairs of lateral leaflets (Figure 3C). Leaves of *trifoliate* homozygote plants have long petioles and bear only one pair of lateral leaflets (Figure 3D).

The *35S::Kn1* transgene was introduced into *La*/+ mutant plants by transformation and by crossing with the transgenic plant M1 shown in Figure 1C. Results from both experiments were identical. With the exception of leaf ramification, *La*/+ plants expressing *35S::Kn1* display all facets of *Kn1* misexpression: leaves are much smaller, sometimes relatively wider, slightly lobed, and frequently rumpled (Figure 3F); growth of *La*/+; 35S::Kn1 plants is severely retarded and apical dominance is lost. *La*/+; *35S::Kn1* plants are fertile and, among progeny of a *La*/+; *35S::Kn1* (M1) plant, *La*/+::+/+; *La*/+; *35S::Kn1*; and +/+; *35S::Kn1* phenotypes segregate in the expected proportions. Thus, the failure of *La*; *35S::Kn1* leaves to subdivide is not attributed to failure in the transcriptional expression of the *35S:Kn1* transgene. Presumably, the meristematic deficiency associated with the *Lanceolate* allele cannot be rescued by *Kn1* ectopic activity and leaf ramification remains arrested in these plants.

In contrast with *La*/+ plants, *Pts/Pts* primary transformants expressing the *35S::Kn1* are not readily distinguishable from wild-type plants possessing the *35S::Kn1* construct. Furthermore, one half of the F1 progeny of *Pts/Pts* plants pollinated by transgenic plant M1 also form supercompound leaves, with multiple diminutive leaflets typical of the parent plant. Leaves of approximately one quarter (6 out of 26) of F2 plants exhibit extreme growth retardation and leaflets with very narrow blades, in addition to being supercompound. Thus, the dominant *Pts* allele neither antagonizes nor enhances the ramification effect of *Kn1*.

The *Lanceolate* leaf is simple and also differs from wild type in that its margins are entire (compare Figure 3E with 1A). To determine which of these two features prevents the ramification effect of *Kn1*, the *35S::Kn1* transgene was introduced into *potato-leaf* (*c*) mutant plants. Leaves of *c/c* plants are compound, but their margins are entire rather than dentate, and they bear only two pairs (rather than three to four) of leaflets (Figure 3C). As shown in Figure 3G, such leaves clearly respond to misexpression of *Kn1* by increased subdivision, as do regular compound leaves, but ramification is restricted to the terminal portion of the midveins, leaving relatively long naked petioles with no or only a slender lamina.

The pattern of the ramified leaves in wild-type, *Pts*, and *potato-leaf* plants suggested that *Kn1* will elicit the multiplication of preexisting compound patterns, but is unable to increase the complexity of a given phyllomere or to rescue the basic compound prototype in mutant plants. To determine the relation between alterations of the basic prototype and additional ramifications further, and to examine the possibility to manipulate leaf architecture in a predictable manner, the *35S::Kn1* transgene was introduced via regular crosses into *trifoliate* mutant plants. A *trifoliate (tf/tf)* leaf of plants expressing one dose of the *35S::Kn1* transgene is shown in Figure 3H. In such leaves, every appendage is converted into a ternate design itself so that three triplets rather than one, and nine leaflets rather than three, are formed (compare with Figure 3D). The wild-type prototype, though, was not restored.

The Tomato *Kn1* Gene: Sequence, Analysis, Genetic Mapping, and Developmental Expression

To explore the possibilities that either *Pts* or *La* may represent mutations in the *Kn1* ortholog of tomato and that the developmental expression of *Kn1* genes in species with simple and compound leaves are different, we

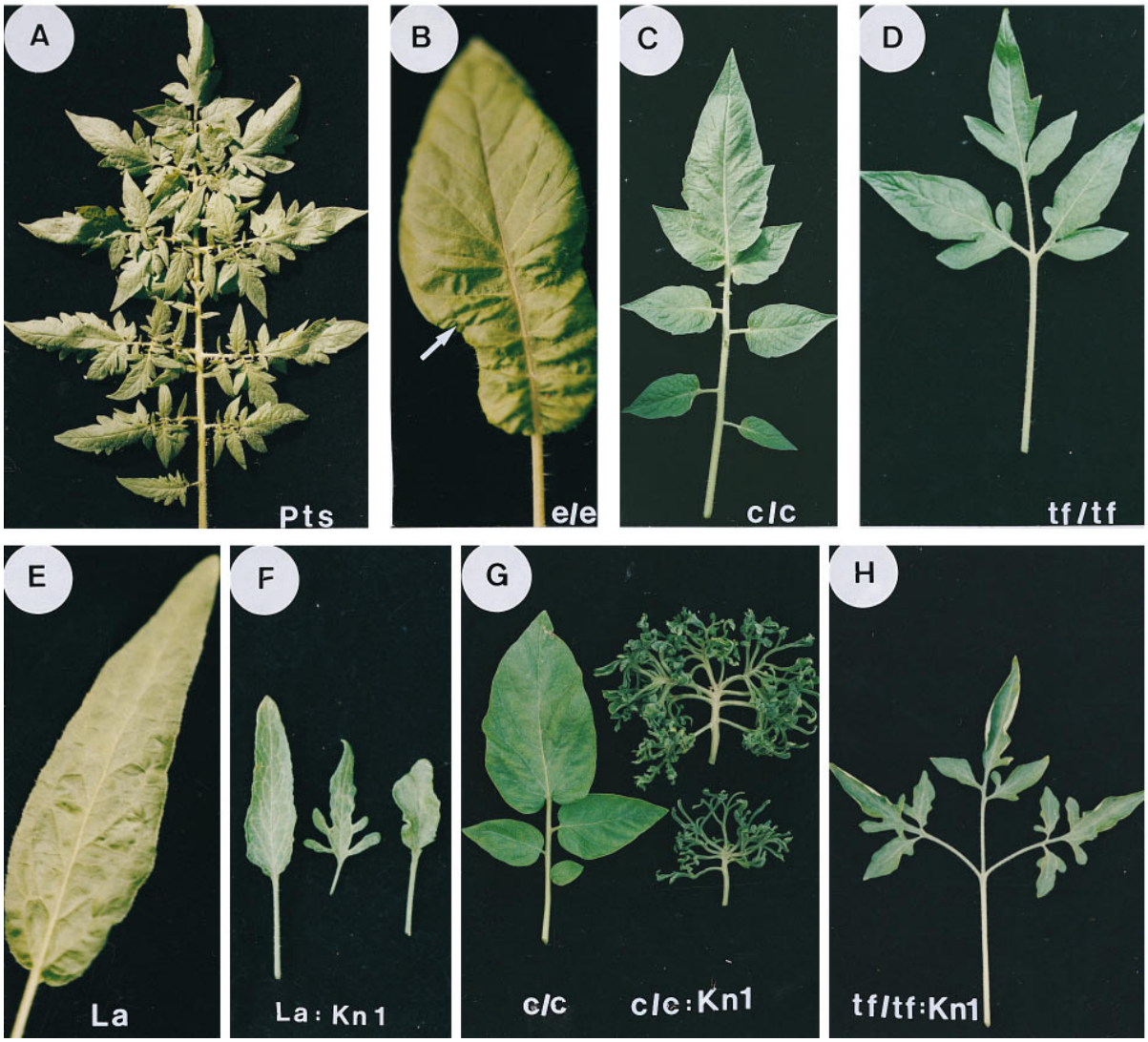

Figure 3. Phenotypic Expression of *Kn1* in Leaf Arrangement Mutants of Tomato

(A–E) Genes affecting leaf compoundness in tomato. (A) Petroselinum. Chromosome VI. Note the additional order of subdivision in comparison with the wild-type prototype shown in Figure 1A. The terminal and lateral leaflets acquire the architecture of the wild-type leaf phyllomere. (B) A pseudosimple leaf of plants homozygote for the *entire (e)* recessive gene. One or more pair(s) of leaflets is merged (or fused) with the terminal leaflet, as suggested also by the altered orientation of veins at the distal half of the structure. The borderline between the terminal leaflet and the fused laterals is indicated by an arrow. Depending on genetic background, less and more extreme leaf arrangements are formed. Chromosome IV. (C) Potato-leaf. Chromosome VI. Only two pairs of lateral leaflets with entire margins are formed. Number of folioles is also reduced. (D) *trifoliate*. Chromosome V. Only terminal leaflets and the most adjacent pairs of laterals are formed. (E) Simple and entire leaf of *Lanceolate* heterozygote (*La*/+) plants. Chromosome VII.

(3F–G) Phenotypic expression of *Kn1* in *La*, *potato-leaf*, and *trifoliate* mutant plants. (F) *Kn1* does not rescue the compoundness of the simple *La*/+ leaf. Three modified leaves of *La*/+; *Kn1* are shown. Note the reduced size and altered lamina shape and compare with *Tobacco::Kn1* leaves in Figure 7B and Sinha et al. (1993).

(G) *Kn1*-induced ramification of *potato-leaf* leaves. Left: young *potato-leaf* with entire margins and only one pair of major leaflets. Right: a ramified *c/c::Kn1* leaf (top) and excised subdivided lateral leaflet (bottom). (H) *trifoliate::Kn1* leaf. Each of the three appendages of the ramified leaf acquires the ternate arrangement and elongated petiole of the progenitor leaf. Compare with Figure 3D.

undertook the isolation of the tomato *Kn1* (*TKn1*) gene. Using the maize gene as a probe, a cDNA clone, (designated *TKn1*), with extensive homology in the homeodomain and flanking sequences was isolated from a tomato shoot cDNA library (Figure 5). All features that characterize the *Kn1*-type homeodomain (for reviews, see Kerstetter et al., 1994; Lincoln et al., 1994; Ma et al., 1994, are conserved in the *TKn1* gene (see Figure 5).

Southern blot analysis (data not shown), screening by various procedures, and subsequent isolation and sequencing revealed that at least five genes belong to the *Kn1* family of tomato. They do not cross-hybridize under stringent conditions, and only *TKn1* exhibits extensive homology outside the homeodomain with the maize and Arabidopsis *Kn1* genes. Restriction Fragment Length Polymorphism. (RFLP) mapping using the N-terminal half of the *TKn1* gene unambiguously placed it on chromosome IV rather than chromosomes VI or VII,

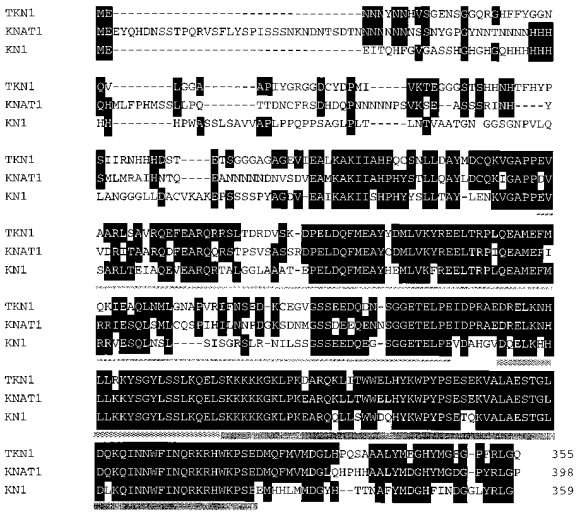

Figure 5. The Tomato *Kn1 (Tkn1)* Gene

Bold and wavy underline, respectively, mark the conserved homeodomain and ELK that characterize all *Kn1* class 1 genes. Note the extensive homology in the 100 residues long presumptive acidic region immediately upstream of the ELK domain. Within this acidic domain most hydrophobic positions are also conserved. The N-terminal one third of the genes is the most variable, but reveals common features as well. It is extremely histidine-rich in maize and soybean, and less so in tomato. It is also dominated by hydrophilic residues: asparagine repeats in Arabidopsis, excess serine and asparagine in soybean, and a very high proportion of glycine in tomato. Identical residues are shaded, and a more detailed analysis of homeodomains of *Kn1* genes is provided in Kerstetter et al. (1994) and Ma et al. (1994).

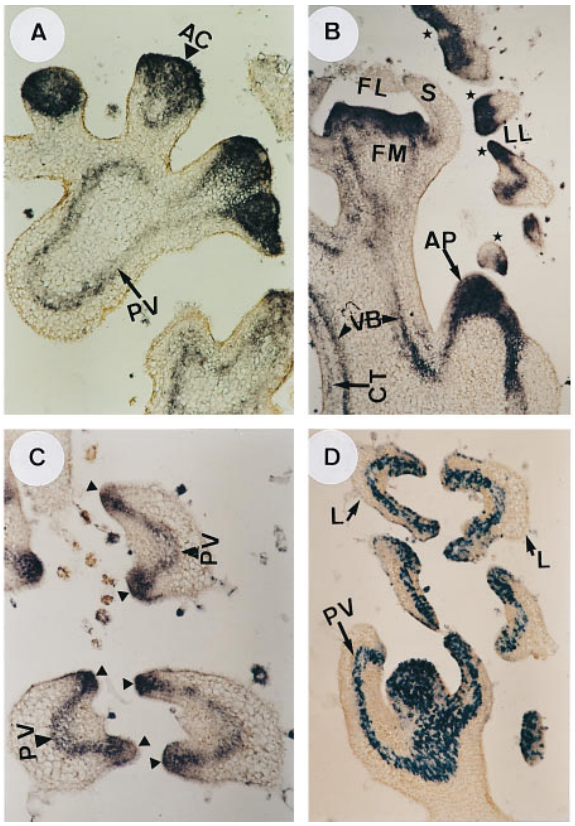

Figure 6. *TKn1* Expression in Leaf and Floral Primordia of Tomato: In Situ Hybridization of DIG-Labeled Antisense Probes

(A) Anantha floral meristems. (Longitudinal sections). Apical cells (AC) and provascular bundles (PV) are labeled.

(B) Shoot apex. A longitudinal section of a floral bud (FL) is shown to the left, and the next sympodial apex (AP) to the right. Stars on the right mark tangential section through lateral leaflets (LL) of the emerging compound leaf. In the floral bud, the floral meristem (FM) and the vascular bundles (VB) are heavily labeled. *TKn1* RNA is found also in the parenchyma cells of the cortex (CT). In the emerging sympodial apex (AP), the apical cells and provascular derivatives are marked and the growing points and provascular strands of newly emerged lateral leaflets are also labeled.

(C) Cross-sections of leaf primordia. *TKn1* transcripts are found in the lateral tips that will form the lamina (arrowheads) and in the provascular tissue (PV).

Complete cDNA and a 477 bp BamHI–HindIII fragment from the 5′ end of the gene give identical distribution of signals.

(D) Expression of the dUTPase gene in leaf primordia and shoot apex. AC, apical cells; CT, cortex; FM, floral meristem; FP, floral primordia; GE, growing ends of leaves; IM, inflorescence meristem; LT, lateral leaflets; PV, provascular strands; S, sepals; SA, shoot apex; VB, vascular bundles.

where *Pts* and *La*, respectively, reside. Mapping with the homeodomain alone gave identical results. No dominant leaf mutants are known to be linked to chromosome IV, but at least six recessive mutations that alter leaf development are located on this chromosome (Stevens and Rick, 1986). The nearest gene to *TKn1* is *entire* (Figure 3B), but *TKn1* cDNA clones originated from *entire* plants were found to be identical at DNA sequence level to the wild-type gene.

As shown in Figure 4B, *TKn1* transcripts were not observed in samples isolated from 3 cm and 5 cm long leaves (lanes 1 and 2), but were found in 0.5 cm long leaves (lane 3). *TKn1* mRNA is abundant in 0.5 cm long shoot apices that carry leaf and floral primordia (lane 4), and more so in the upper part of the stems when stripped of these appendages (lanes 5 and 6). Low levels of mRNA are found in mature flowers (lane 7), due probably to the floral pedicles and carpels (lane 11), and in arrested floral meristems of the *anantha* mutant inflorescences (lane 12). *TKn1* is also expressed at the wild-type level in shoot apices of *La*/+ plants (data not shown).

Since *Kn1*-related genes are not expressed in leaf and floral primordia of maize or Arabidopsis (Lincoln et al., 1994; Kerstetter et al., 1994), in situ hybridization was used to localize more precisely the *TKn1* transcripts in these organs in tomato plants (Figure 6). In the floral meristems of *anantha* mutant plants (Figure 6A), which are arrested in the preorganogenesis stage (Helm, 1951; Pri-Hadash et al., 1992), *TKn1* is expressed in all layers of the apical meristem AC and in the provascular (PV) strands. The sympodial shoot of tomato is composed of reiterated units of three leaves and a terminal inflorescence. In Figure 6B, a longitudinal section of a floral bud is shown to the left, and a tangential section cutting through a series of lateral leaflet primordia of a leaf on the right. Evidently, leaflet primordia (stars) and the meristematic zone of the next sympodial apex are stained. Staining is strong in the meristematic (FM) region of the future inner three whorls of the floral bud, but it is very weak in the emerging sepals (S). *TKn1*

transcripts were detected in the newly emerged lateral primordia (LT) in the floral bud and their vascular bundles (VB), and in the cortex parenchyma (CT) of the floral pedicle. A more accurate picture of the localization of *TKn1* transcripts in leaf primordia is obtained from the cross sections shown in Figure 6C, where provascular strands and lateral growing tips (arrowheads) are labeled. The internal growing tips give rise to the lamina, upon primordium expansion. This pattern is practically identical to that of the dUTPase gene in the very same organs (Figure 6D; Pri-Hadash et al., 1992).

The Weak Meristem- and Vascular-Specific dUTPase Promoter Is Sufficient to Elicit the *Kn1* Syndrome in Tomato

The ramification of leaves of transgenic *35S::Kn1* tomato plants is apparent very early in the development of the leaf primordium (see Figure 2). We have used the recently isolated promoter of the tomato dUTPase gene to examine the role of the meristematic and provascular cells of the leaf primordium in determining the subdivision of the compound leaves. Similar to the *TKn1* gene, the dUTPase gene functions predominantly in apical meristems of vegetative and floral organs, as well as in provascular cells with meristematic potential (Figure 6; Pri-Hadash et al., 1992). It is down-regulated in the parenchyma derivatives and other differentiated tissues, and its expression in mature leaves (5 cm long) or mature flowers is negligible. A 380 bp proximal sequence of the putative 5′ regulatory region of the dUTPase gene was shown to drive the expression of the β-glucuronidase reporter gene in the above tissue domains (O. Cohen, unpublished data) and was used in the experiments reported here.

We generated 31 kanamycin-resistant primary transformants expressing *Kn1* under the control of the dUTPase promoter (designated B series), and leaf ramification, as illustrated in Figure 7A, was observed in 21 plants. An important feature of this series of transgenic plants is that the extent of leaf ramification varied from one transformant to another; this was not evident among the M series plants. Leaves of transgenic plant B1, for example, are subdivided only once more and, in this respect, precisely mimic the arrangement of leaves in plants bearing the dominant *Pts* gene shown in Figure 3A. One additional order of subdivision is exhibited by plant B100 and more extreme ramifications by other B series plants. These results, along with those obtained with the *trifoliate::Kn1* plants, illustrate our ability to manipulate at will the architecture of the compound leaf. The expression of the *PdUTPase:Kn1* transgene in transformed tomato plants is compared with that of the *35S::Kn1* transgene in Figure 4A (lanes 5–8). *Kn1* transcripts are hardly detected in the B1 plant with the very weak phenotypic response, and they are also rare, in comparison with the *35S::Kn1* transformants, in other B series plants that manifest full leaf ramification. Misexpression of *Kn1* in the dUTPase territories is sufficient, therefore, to induce the full potential of leaf ramification. It does not imply, by any means, that expression of *Kn1* in mesophyll or epidermal cells will not result in altered morphogenesis.

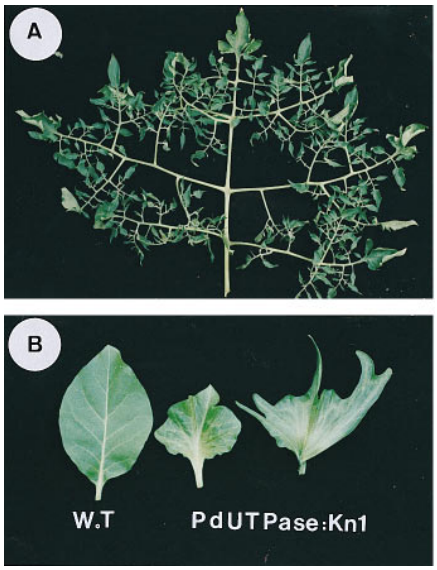

Figure 7. The Vascular-Specific dUTPase Promoter Is Sufficient to Elicit Leaf Ramification.

(A) A compound leaf of tomato transgenic plant expressing the *PdUTPase::Kn1* transgene. Leaves of such transgenic plants are distinguished from *35S::Kn1* plants by the longer petioles of their lateral leaflets and by the prominence of the terminal leaflet in each compound unit.

(B) Wild-type (left) and highly lobed leaves of transgenic tobacco plant expressing the *PdUTPase::Kn1* transgene. This phenotype is similar to that reported for *35S::Kn* tobacco plants (Sinha et al., 1993).

To find out whether the effect of the *PdUTPase::Kn1* transgene is species specific or, like that of the *35S::Kn1*, depends on the developmental status of the leaf, we have introduced it into tobacco plants as well. Indeed, as shown in Figure 7B, we obtained transformed kanamycin-resistant plants, with modified leaves (7 out of 12), similar to those reported in *35S*-driven expression in transgenic tobacco (Sinha et al., 1993).

The overall 50-fold difference in the expression of the *35S::Kn1* and *dUTPase::Kn1* transgenes is not reflected in the severity of the phenotypes in tobacco or in the degree of subdivision in tomato. It is the expression in particular cells at particular times that matters most.

Discussion

A General Scheme for Leaf Morphogenesis

Fortuitously, leaves of all species in which misexpression of *Kn1* was previously examined (i.e., maize, tobacco, rice, and Arabidopsis) were simple. We have shown here that misexpression of *Kn1* affects compound leaves of tomato in a very different way than it affects simple leaves and that this basic observation reflects inherent fundamental differences in the development of simple and compound leaves. This hypothesis implies that simple and compound leaves are determined by two different developmental programs and that the gene systems that condition them are conserved among species with simple and compound leaves, respectively.

An attempt to develop an experimental framework for the genetic dissection of leaf arrangement requires the formulation of a likely developmental scenario, a judicious classification of the many genes involved, and their isolation and characterization. Formally, the formation of the prototype compound unit of tomato entails the establishment, in a regular basipetal pattern, of pairs of lateral meristems along the peg-like structure of the primary dorsiventral primordium, and the concomitant inhibition of lamina expansion. Additional orders of subdivision require the reiterated formation of lateral meristems on the secondary primordia before lamina expansion ensues. We speculate that the interplay between apical growth, formation of lateral meristems, and activation of the diffused leaf meristems that condition the expansion of the lamina determine whether a leaf will be compound or simple. As long as the primordium apex continues to "grow," emanating signals that induce the formation of lateral meristems, and a coupled system delays the activation of the diffused meristem, the leaf will be compound. Maintaining this balance among the developmental programs allows for more subdivisions to ensue. Presumably, an additional, species-specific developmental plan dictates the developmental window in which "active" cell types, i.e., apical, provascular, and lateral lamina, are allowed to proliferate.

The inherent inability of simple leaves to respond properly to *Kn1* overexpression is reflected by ectopic formation of sheath tissues and "knots" in blade territories in maize (Sinha and Hake, 1994) and in irregular expansion of the lamina and the ectopic formation of shoots on tobacco and Arabidopsis leaves (Sinha et al., 1993; Lincoln et al., 1994). The developmental requirements for compoundness cannot be satisfied under the restrictions imposed by the "simple" program, and the *Kn1* gene product that enhances meristematic activity in leaves affects only secondary growth parameters, resulting in a variety of malformations. The reiterated ramification of the compound prototype unit of the tomato leaf is, according to this view, due to developmental programs that distinguish compound from simple leaves.

Genetic Evidence for the Developmental Program Controlling Compoundness

All aerial meristems have a common onthogenetic origin. Consequently, mutations in basic functions of meristems, as amply illustrated by *Kn1*, are expected to have multiple pleiotropic effects. The actual most prominent manifestation of such mutations will depend on local developmental programs. In accord with the proposed developmental scenario, we suggest that the recessive *potato-leaf* and *trifoliate* mutations, as well as the dominant *Pts* and *Kn1* alleles, modify preferentially the balance between lamina expansion and lateral leaflet meristems. The *La* dominant mutation subdues, preferentially, meristematic activities without which the potential for compoundness, and thus ramification, cannot be materialized.

potato-leaf and *trifoliate* reduce the complexity of the compound phyllomer but are not involved directly in the plan that permits compoundness. *trifoliate* is more extreme, allowing only one pair of lateral leaflet meristems. In the less extreme potato leaf, however, where two pairs of leaflets are formed, the blades are entire rather than dentate, suggesting that the two genes affect meristematic functions in different ways. The lesions in both cases, we surmise, favor early lamina expansion and, consequently, also limit to a certain extent the ability to subdivide in response to *Kn1*.

The *Pts* dominant mutation, most likely an overproducer allele, shifts the balance in the opposite direction; lamina growth is delayed for one additional cycle with no associated alteration in the complexity of the phyllomer. By the same token, the dominant effect of *Kn1* results in extra ramification with no increase in the number of lateral meristems in each unit, and *Kn1* fails to rescue the compound prototype of *potato-leaf, trifoliate*, and probably *entire* as well. Thus, *Kn1* can only ramify a preexisting plan as it allows the proliferation of active cell type during a given developmental window. In so doing, *Kn1* dismisses the fine-tuning conferred by the wild-type *Pts* system, and it is not unrealistic to suggest that the endogenous *TKn1* gene regulates the activity of *Pts* in leaf primordia.

La is epistatic to all known leaf shape mutations (E. Lifschitz, unpublished data), and in addition, arrest meristematic activity in all aerial meristems. *La/La* embryos produce root meristems, but fail to develop shoot apical meristems (Mathan and Jenkins, 1962; Caruso, 1968; Dengler, 1984); the shoot apical meristems are "normal" in *La/+* plants. *La/+* fruits are the size of small cherry tomatoes, and meristematic activity in leaves is differentially reduced, preventing the formation of lateral meristems and dentate margins, but not of the appearance of the primary leaf primordium itself. From the dose effect studies of Stettler (1965) in tetraploid plants, we infer that *La* is an antimorph or a neomorph, rather than a haploinsufficient or an overproducer. *La/+* leaves are morphogenetically simple and thus similar to Arabidopsis and tobacco leaves. They remain simple following misexpression of *Kn1*, albeit displaying the other aspects of the *35S::Kn1* phenotype, just like leaves of Arabidopsis and tobacco. *Pts* and *Kn1* fail to rescue the compound prototype or to induce ramification of *La/+* leaves. The response of simple leaves of tomato (i.e., *La*), Arabidopsis, and tobacco to *Kn1* is thus species independent, which suggests that they are developmentally, and not merely morphogenetically, in the same state.

We suggest expanding the analogy between *La* leaves and simple leaves to sepals as well. Sepals of tomato, tobacco, and Arabidopsis are also simple and entire, and similar to simple leaves, sepals do not respond to *Kn1* misexpression by any elaboration of their form, but their venation pattern is distorted and the areole size is increased. In contrast with sepals, the potential for ramification is silent but otherwise intact in tomato juvenile leaves. Juvenile leaves of tomato normally have only one to three leaflets, but they ramify to the same extent proportionally, as adult leaves do upon ectopic expression of *Kn1*.

The phenotypic manifestation of *Kn1* misexpression can thus be used to verify the inherent nature of leaf architecture. It is possible, for example, that in some

plant species, compound leaves are actually modified simple leaves (i.e., Kaplan, 1983). In this context, it will be interesting to see the fate of tendrils and leaflets in pea, or to find out whether the basic ternate arrangement in clover leaves will be converted to multiples of three following misexpression of the *Kn1* gene.

The Possible Role of Apical and Provascular Cells of the Primary Dorsiventral Primordium in the Formation and Reiteration of the Compound Prototype

The difference between tomato and the species with simple leaves is that the *Kn1*-induced meristematic activity in tomato is exploited very early during primordia development for the ramification of the compound phyllomer. The knots in maize, and the malformations in the leaves of transgenic *35S::Kn1* tobacco and Arabidopsis plants, are not visibly detected until relatively late in leaf development (Sinha et al., 1993; Lincoln et al., 1994). To understand the formation of compound leaves in tomato, we will have to identify the target cells for the *Kn1*-induced ramification.

The ectopic expression of the dominant mutation *Kn1* in maize is restricted to vein tissues (Smith et al., 1992), but the *35S*-driven expression in tobacco, Arabidopsis, and tomato is ubiquitous. We do not, therefore, know what the phenotypic consequence of *Kn1* misexpression outside the vasculature will be, or, expression in which tissue is responsible for the variety of pleiotropic effects seen in the different species.

Expression of *Kn1* driven by the meristem and provascular-specific PdUTPase promoter was shown to induce the full potential of leaf ramification in tomato (Figure 7) and the loss of apical dominance as well. While it is possible to relate the formation of laterals to differential localization of hypothetical KNOTTED signals along the provascular strands of the dorsiventral leaf primordia, it is impossible to exclude a role for the primordium apical cells. Apical cells were not expected to respond to misexpression of *Kn1,* since the *Kn1* gene was shown to express normally in apices of shoots and inflorescences, and its overexpression there had no obvious morphogenetic consequences in tobacco or Arabidopsis (Smith et al., 1992; Jackson et al., 1994; Sinha et al., 1993; Lincoln et al., 1994).

That apical cells of the dorsiventral leaf primordium are required for the initiation of the compound leaf is supported by the expression pattern of *TKn1* in the plant meristems (Figure 6). Expression of all *Kn1*-subclass genes in Arabidopsis and maize is clearly excluded from leaf and floral primordia (Lincoln et al., 1994; Kerstetter et al., 1994). The observation that tomato and Arabidopsis display different expression patterns of homologous regulatory genes in their respective meristems is not surprising, however, and conceptually similar to what was described for the homeotic genes in the animal kingdom (see Carroll, 1995). The two dicot plant species must employ homologous regulatory genes in order to execute contrasting meristematic programs: monopodial shoots, indeterminate inflorescences, and simple leaves in Arabidopsis; sympodial shoots, determinate inflorescences, and compound leaves in tomato (Hareven et al., 1994). While this by itself does not constitute evidence that expression of *TKn1* in the leaf primordium is required for the formation of the compound structure or for its ramification, the induction of additional subdivisions by the PdUTPase-driven *Kn1* expression in the same domains where *TKn1* is expressed supports such a premise.

While the overall scenario suggested here for the formation of compound leaves is open to question, there is no doubt that leaf morphogenesis is presently open to molecular and genetic dissection. Since no other organ of living creatures exploits analogous sets of genes to acquire such a developmental flexibility and to generate such a range of diverse forms both between and within species, understanding the underlying mechanisms will be of fundamental importance.

Experimental Procedures

Tomato Lines

The following mutant lines were provided by Prof. C. M. Rick (University of California, Davis): *anantha (an)* LA536; *entire (e)* LA2922; *Lanceolate (La) LA335;* potato-leaf (c) LA3211; *self-pruning (sp)* LA154; *Petroselinum (Pts)* LA2532; *trifoliate2 (tf2)* LA579. VF36 seeds were the gift of S. McCormick, PGEC, Albany. Indeterminate line 93–137 (*Sp/Sp*) was provided by D. Zamir (Rehovot).

Cytological Procedures

In situ hybridization using digoxygenin-labeled RNA probes were according to J. H. Doonan and E. Coen (personal communication) and Jackson (1991), with the following minor modifications: buffer 1 was 100 mM maleic acid 150 mM NaCl (pH 7.5), as recommended by Boehringer. Dilution of antidigoxygenin-AP of Boehringer was 1:1000 and incubation time with the antibody was 1.5 h.

Observations of venation patterns were conducted on cleared leaves following 5 min boiling in 85% ethanol and 3 h in lactic acid at 70°C. Preparation of tissues for SEM were performed according to a protocol provided by R. Meeks-Wagner (personal communication).

Nucleic Acids Procedures

The maize *Kn1* cDNA clone (Volbrecht et al., 1991) in the pBIN19 vector (Bevan, 1984) was provided by S. Hake. The *Kn1* gene driven by the 380 bp PdUTPase promoter (O. Coen, unpublished data) was also cloned into the same binary vector, and transformations of both were performed via Agrobacterium tumefasciens strain LBA4404 in the RK9/8 line (Pnueli et al., 1994b) according to Horsch et al. (1985) or in VF36 according to McCormick (1991).

The screen for the *TKn1* gene was conducted with the maize *Kn1*-labeled cDNA under relaxed hybridization conditions: 15% formamide, 5× SSC, 5× Denhardt's solution and 1% SDS in 42°C. Filters were washed with 3× SSC, 1% SDS in 37°C and exposed for 48 hr. The shoot cDNA library representing 1.2×10^6 independent inserts was constructed in the λZAPII vector (Stratagene) from the second and third sympodial shoots of the indeterminate line 93–137. The 5 mm long apices contain the apex, three to four leaf primordia, and one to three floral primordia in which only sepals emerged. Other nucleic acid procedures followed published protocols (Ausubel et al., 1988).

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