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Reduced leaf complexity in tomato wiry mutants suggests a role for *PHAN* and *KNOX* genes in generating compound leaves

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SUMMARY

Recent work on species with simple leaves suggests that the juxtaposition of abaxial (lower) and adaxial (upper) cell fates (dorsiventrality) in leaf primordia is necessary for lamina outgrowth. However, how leaf dorsiventral symmetry affects leaflet formation in species with compound leaves is largely unknown. In four non-allelic dorsiventrality-defective mutants in tomato, *wiry, wiry3, wiry4* and *wiry6*, partial or complete loss of ab-adaxiality was observed in leaves as well as in lateral organs in the flower, and the number of leaflets in leaves was reduced significantly. Morphological analyses and expression patterns of molecular markers for ab-adaxiality [*LePHANTASTICA* (*LePHAN*) and *LeYABBY B* (*LeYAB B*)] indicated that ab-adaxial cell fates were altered in mutant leaves. Reduction in expression of both *LeT6* (a tomato *KNOX* gene) and *LePHAN* during post-primordial leaf development was correlated with a reduction in leaflet formation in the wiry mutants. *LePHAN* expression in *LeT6* overexpression mutants suggests that *LeT6* is a negative regulator of *LePHAN*. *KNOX* expression is known to be correlated with leaflet formation and we show that *LeT6* requires *LePHAN* activity to form leaflets. These phenotypes and gene expression patterns suggest that the abaxial and adaxial domains of leaf primordia are important for leaflet primordia formation, and thus also important for compound leaf development. Furthermore, the regulatory relationship between *LePHAN* and *KNOX* genes is different from that proposed for simple-leafed species. We propose that this change in the regulatory relationship between *KNOX* genes and *LePHAN* plays a role in compound leaf development and is an important feature that distinguishes simple leaves from compound leaves.

Key words: KNOX, PHAN, Tomato, Leaf dorsiventrality, Compound leaf

INTRODUCTION

In higher plants, the shoot axis is radially symmetrical while lateral organs such as leaves have asymmetric features. This asymmetry is visible along three axes of the organ; proximodistal (base to tip), mediolateral (midrib to margin) and adaxial-abaxial (upper and lower). In most higher plants the upper (adaxial) part of the leaf is anatomically and physiologically different from the bottom (abaxial) part of the leaf. This asymmetry (ab-adaxiality) is established early in the leaf primordium and the shoot apical meristem (SAM) seems to provide positional cues for the initial establishment of this asymmetry (Hanawa, 1961; Lynn et al., 1999; Snow and Snow, 1959; Sussex, 1954; Sussex, 1955; Timmermans et al., 1998). Ab-adaxiality defective mutants have been reported in *Antirrhinum* (Waites and Hudson, 1995), *Arabidopsis* (Bohmert et al., 1998; Bowman and Smyth, 1999; Chen et al., 1999; Eshed et al., 2001; McConnell and Barton, 1998; Sawa et al., 1999b; Siegfried et al., 1999), maize (Freeling, 1992; Timmermans et al., 1998), *Nicotiana* (McHale, 1993a; McHale, 1993b; McHale and Marcotrigiano, 1998), tomato (Kessler et al., 2001) and pea (Meicenheimer et al., 1983).

Several genes in several species are thought to specify the adaxial and abaxial domains. For example, leaf adaxial cell fate is replaced by abaxial cell fate in the *phantastica* mutation of *Antirrhinum*, suggesting that *PHANTASTICA* (*PHAN*), a MYB domain transcription factor, plays an important role in establishing (or maintaining) adaxial cell fate in leaf primordia (Waites and Hudson, 1995; Waites et al., 1998). In *Arabidopsis ARGONAUTE1* (*AGO*), *REVOLUTA* (*REV*) and *PINHEAD* (*PNH*) are also important for specifying adaxial cell fate in lateral organs and for promoting meristematic activity in the SAM and axillary meristems (Bohmert et al., 1998; Lynn et al., 1999; Talbert et al., 1995). PHABULOSA and PHAVOLUTA are homeodomain-leucine zipper (HD ZIP III) proteins with a START (steroid/lipid-binding) domain expressed in the adaxial cells of the leaf primordium and in the SAM and semi-dominant mutations in these genes produce radial leaves with

adaxial cell fates (McConnell and Barton, 1998; McConnell et al., 2001). In the *leafbladeless* mutant in maize, ectopic patches of abaxial identity are seen on the adaxial side of the leaf and ectopic lamina forms at the boundary between the two cell fates (Timmermans et al., 1998). *FILAMENTOUS FLOWER* (*FIL*), *YABBY2* (*YAB2*), *YABBY3* (*YAB3*) and *KANADI* are expressed only abaxially in all lateral organs of *Arabidopsis*, and ectopic expression of *FIL* or *YAB3* is sufficient to induce ectopic abaxial patches in the adaxial region of the leaf (Sawa et al., 1999a; Siegfried et al., 1999). Together, all these mutant phenotypes strongly suggest that the juxtaposition of adaxial and abaxial cell fates is necessary for proper leaf lamina development in simple-leafed species, and that adaxial and abaxial cell fates are mutually antagonistic.

The Class I *KNOTTED-1 LIKE HOMEOBOX* (*KNOX I*) genes play an important role in maintaining indeterminacy in the SAM and in subsequent shoot development. Loss-of-function mutations in some of these genes (e.g. *kn1* and *stm*) result in an inability to form or maintain a SAM (Barton and Poethig, 1993; Kerstetter et al., 1997; Smith et al., 1995; Vollbrecht et al., 2000). Mutations in other *KNOX* genes cause reduced internode or axis elongation (Postma-Haarsma et al., 2002; Venglat et al., 2002). Ectopic overexpression of *KNOX* genes in dicots leads to more dissected and highly lobed leaves, often accompanied by ectopic shoot meristem formation on leaves (Chen et al., 1997; Chuck et al., 1996; Janssen et al., 1998; Lincoln et al., 1994; Nishimura et al., 2000; Sinha et al., 1993). Dominant mutants in the *KNOX* gene *LeT6*, *Mouse Ears* (*Me*) and *Curl* (*Cu*), express *LeT6* ectopically in the mature leaves and show increased leaf dissection (Chen et al., 1997; Parnis et al., 1997). Furthermore, *KNOX* gene expression in leaf primordia accompanies leaf dissection in many species, suggesting a role for *KNOX* genes in making compound leaves (Bharathan et al., 2002).

PHAN is reported to be a negative regulator of *KNOX* genes. Mutations in *PHAN* orthologs (*RS2* in maize and *AS1* in *Arabidopsis*) caused *KNOX* genes to be expressed ectopically (Byrne et al., 2000; Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). The phenotype of the double mutant, *stm/stm, as1/as1* indicates that *as1* is epistatic to *stm* in *Arabidopsis* (Byrne et al., 2000). Because *AS1* represses *KNAT1* (and *RS2* represses *RS1*) and *STM* in turn represses *AS1*, the expression domains of *PHAN* orthologs and *KNOX* genes do not overlap (Byrne et al., 2000; Timmermans et al., 1999; Tsiantis et al., 1999; Waites et al., 1998). *PHAN* orthologs are expressed only in the incipient leaf primordium (P_0) and developing leaf primordia (Timmermans et al., 1999; Tsiantis et al., 1999; Waites et al., 1998), but *KN1* and *STM* are expressed in the SAM and are downregulated in P_0 and leaf primordia in species with simple leaves (Jackson et al., 1994; Long et al., 1996). However, in tomato *LePHAN* and *LeT6* mRNA were both detected in the SAM, in leaflet primordia and in growing leaflet laminas (Chen et al., 1997; Janssen et al., 1998; Koltai and Bird, 2000).

We describe four non-allelic mutants, *wiry* (*w*), *wiry3* (*w3*), *wiry4* (*w4*) and *wiry6* (*w6*) that are defective in ab-adaxial symmetry in tomato. The degree of leaf compounding in these mutant plants was severely reduced. The expression patterns of *LeT6*, *TKN1*, *LePHAN* and *LeYAB B* were determined in the *w*, *w3* and *w6* mutants. The regulatory relationship between *LePHAN* and *KNOX* genes in the meristem and early leaf primordium is different from that seen during the later stages of leaf development in tomato and may explain the compound nature of the tomato leaf.

MATERIALS AND METHODS

Plant material and growth conditions

Homozygous mutant seeds of *w3*, *w6* (Rick and Butler, 1956), *Me* and *Cu* seeds were obtained from the Tomato Genetics Resource Center (TGRC) at the University of California at Davis (accession number: *w3* LA1498 cv. First Early, *w6* LA2065 cv. Rheinland Rhum, *Me/Me* LA0324 cv. Rutgers and *Cu/Cu* LA0325 cv. Stocksdale). For *w* (Rick and Butler, 1956) and *w4* (Clayberg et al., 1966), F_2 seeds of self-pollinated heterozygotes (accession number: *w/+* LA0274 cv. Canary Export, and *w4/+* LA2-237 cv. Pearson) were obtained from TGRC and among the F_2 plants, *w/w* and *w4/w4* plants were examined. Tomato cotyledons of cv. VF36 were transformed according to published protocols (McCormick, 1991) to generate 35SPHAN antisense plants (Kim et al., 2003). All plants were grown in a growth chamber at 22°C with 65% relative humidity and a day length of 16 hours.

Mapping the *LePHAN* locus and *w6*

The *w* and *w4* loci are on chromosome four (at 20 cM and 28 cM from the distal end of short arm). The *w6* locus was mapped using an F_2 mapping population from a cross between *w6* (*L. esculentum*) and *L. pennellii* (Tanksley et al., 1989). Using recombination between the *w6* mutant phenotype and a *LePHAN* RFLP (*Hin*dIII) between *L. esculentum* and *L. pennellii*, we determined that the *w6* locus is 30 cM from the *LePHAN* locus on chromosome 10.

Histology and scanning electron microscopy

Tissues for plastic sections were fixed and sectioned as described previously (Kessler et al., 2001). Samples were viewed with a Nikon Eclipse E600 microscope and images collected using a SPOT (RT Color) digital camera. Samples for SEM were fixed and viewed as described previously (Kessler et al., 2001). Electronic images, collected either directly from the SEM or from a SPOT camera, were processed in Adobe Photoshop.

In situ hybridization and RT-PCR in situ hybridization

In situ hybridizations were performed as described previously (Long et al., 1996) using full-length cDNA probes for *LeT6*, *TKN1*, *LeYAB B* and *LePHAN*. Approximately 500,000 pfu of a λgt10 library from 6-7 mm tomato flowers were screened using *INNER NO OUTER*, a *YABBY* member, as probe (Villanueva et al., 1999) to obtain *LeYAB B*. Median sections (containing the SAM) from multiple different tissue samples including positive controls were placed on each slide and processed. Each experiment was repeated at least four times. Tissues for RT-PCR in situ hybridizations were embedded, sectioned with a Zeiss Microtome HM340E, and processed as previously described (Long et al., 1996). Instead of an overnight hybridization step, RT-PCR was performed on sections as previously described (Ruiz-Medrano et al., 1999). Primers used for the RT-PCR in situ experiments were designed based on the cDNA sequence of *LePHAN* and *LeT6* as follows:

LePHAN1: 5′ACGAGCAGCGTCTTGTTATACAACTAC3′,
LePHAN2: 5′CCCTTCGTCTAAATCCTTGCAGC3′,
LeT65′: 5′TCTTTAACTAACAATAACAATGCAGAAAC3′,
LeT63′: 5′CCAAAGCAGATTCATGAGAAGAATAG3′.

Immunolocalization

Immunolocalization was performed as described previously (Jackson et al., 1994) using a polyclonal antibody against ROUGHSHEATH2 [a generous gift from Dr Marja Timmermans, for details on antibody preparation see Kim et al. (Kim et al., 2003)].

RESULTS

Abaxialization of leaf and reduction of leaflet number in *w*, *w3* and *w6* plants

Wild-type tomato produces unipinnate compound leaves with 7-9 leaflets (Fig. 1A). *w*, *w6* and *w3* plants produced mostly cup-shaped or wire-like leaves, but occasionally produced twisted, irregularly shaped flattened leaves with one or two leaflets (Fig. 1B,C,E). In the compound leaves of the *w*, *w3* and *w6* mutants, there were 27%, 34% and 19.9% leaflets respectively, compared to wild type (100%; Table 1). The incidence of cup-shaped or wire-like leaves increased in later stages of plant development. A unique morphology was often seen in *w3* leaves. These leaves subtended an axillary bud. After production of one or two leaflet pairs, the rachis split and each branch produced an almost complete compound leaf. Often at the junction of the split an axillary-bud like structure was seen (Fig. 1C,D). The *w3* and *w6* mutant plants produced cup-shaped leaves. In contrast, *w* mutants made tendril-like terminal leaflets. The *w*, *w3* and *w6* mutant plants formed normal axillary buds in the axils of the wire-like leaves (Fig. 1F).

To determine if wire-like leaves were produced by abaxialization or adaxialization, the anatomy of these leaves were examined. All parts of a wild-type leaf (including petiole and rachis) have distinct ab-adaxiality. Vascular bundles in the tomato leaf are amphiphloic with both abaxial and adaxial phloem flanking the central xylem (Fig. 2A). Elongated palisade mesophyll cells are located in the adaxial side of the leaf and spongy mesophyll cells are present in the abaxial region of the leaf lamina (Fig. 2B). The *w*, *w3* and *w6* wire-like leaves were radially symmetric (Fig. 2E-H). This anatomy differed both from the wild-type stem, with a cylinder of vascular tissue surrounding a central pith, and from the wild-type petiole, with clear ab-adaxial symmetry (Fig. 2C,D). Vascular bundles of *w*, *w3* and *w6* leaves often had xylem in the center encircled by phloem (Fig. 2E,G,H). Mesophyll cells surrounded the central solid vascular cylinder, but did not have features of distinct elongated palisade mesophyll cells (Fig. 2E-H). In *w3* leaves producing ectopic leaves with axillary buds, the primary rachis, prior to splitting, had an incompletely closed ring-shaped vascular bundle (arrow), suggesting that this leaf is chimeric with features of both the leaf and the stem (Fig. 2F).

Table 1. Leaflet numbers of *wiry* mutants

Mutant	wt	*w*	*w3*	*w6*
Average leaflet number	8.3	2.3	2.9	1.7
s.d.	1.1	1.8	1.8	0.8

n=50 for wild-type and wiry mutant leaves.
s.d., standard deviation

The expanded and flattened leaves of *w3* and *w6* often showed abaxial patches on the adaxial side of the leaf. In these abaxial patches palisade mesophyll cells were replaced by spongy mesophyll cells (Fig. 2I,J). The *w3* and *w6* leaf had a semicircular vascular bundle with the inner phloem clustered at one end on the adaxial side (Fig. 2L,M) rather than a horseshoe-shaped vascular bundle in the midrib as in the wild-type leaf (Fig. 2A). This suggests a reduced adaxial domain in *w3* and *w6* leaves. The flattened *w* leaves had normal mesophyll differentiation in the leaf lamina (Fig. 2K). However, in *w*, vascular bundles in the midrib were reduced and ectopic palisade cells developed on top of the midrib region (Fig. 2N).

Scanning electron microscopy (SEM) revealed that epidermal cell fates were altered in *w3* and *w6* leaves. The adaxial epidermal cells of the wild-type leaf were less lobed with fewer crenulations and very few stomata (Fig. 3A), while the abaxial epidermal cells were highly crenulated and irregularly zigzag-shaped with lots of stomata (Fig. 3B). In addition, the wild-type adaxial leaf surface was smooth, compared to the rougher abaxial leaf surface. In contrast, both the upper and lower epidermal cells of *w3* leaves had characters intermediate between those seen in the abaxial and adaxial surface of wild type. Both epidermal cells were less lobed (like wild-type adaxial epidermal cells) and had more crenulations (like wild-type abaxial epidermal cells) with roughly equal numbers of stomata, suggesting the loss of distinct abaxial-adaxial epidermal differentiation (Fig. 3C,D). In *w6*, epidermal cells on both leaf surfaces were highly crenulated and irregular in shape, suggesting abaxialization of the adaxial epidermis of the leaves (Fig. 3E,F). However, *w* epidermal cells were normal with distinct ab-adaxial features (Fig. 3G,H).

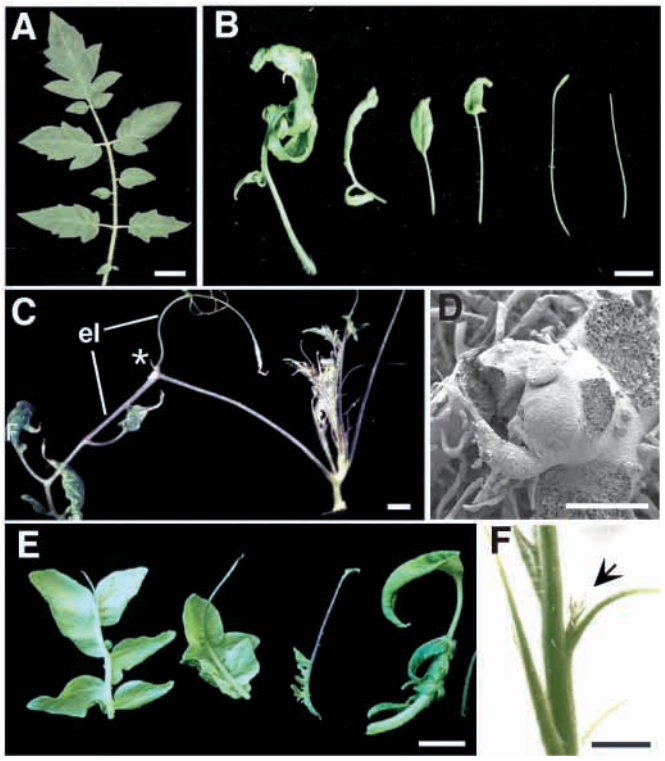

Fig. 1. Leaf phenotypes of *w*, *w3* and *w6*. (A) wild-type unipinnate compound tomato leaf. (B) *w6* plants produce, from the base to the apex, less compound leaves with irregularly shaped blades, cup-shaped leaves and wire-like leaves. (C) *w3* plants with less compound leaves. Often wire-like leaves produce two ectopic leaves (el) distally and a SAM (star) is formed between the junction of these two ectopic leaves. (D) Scanning electron micrograph of the ectopic meristem in C. (E) *w* leaves. (F) Axillary buds on *w6* plants. Scale bars: (A-C,E,F) 1 cm, (D) 250 μm.

Floral organs of *w*, *w3* and *w6* are abaxialized

To see if other lateral organs were also abaxialized, we examined floral organs in *w*, *w3* and *w6*. Tomato flowers have five sepals, petals and stamens and two fused carpels. The bases of sepals are fused into a cup-shaped structure. The corolla is tubular and anthers are adnate to the corolla tube (Fig. 4A). *w*, *w3* and *w6* flowers usually had extra floral organs (e.g. 7-10 sepals and petals) and lacked the fusion of floral organs

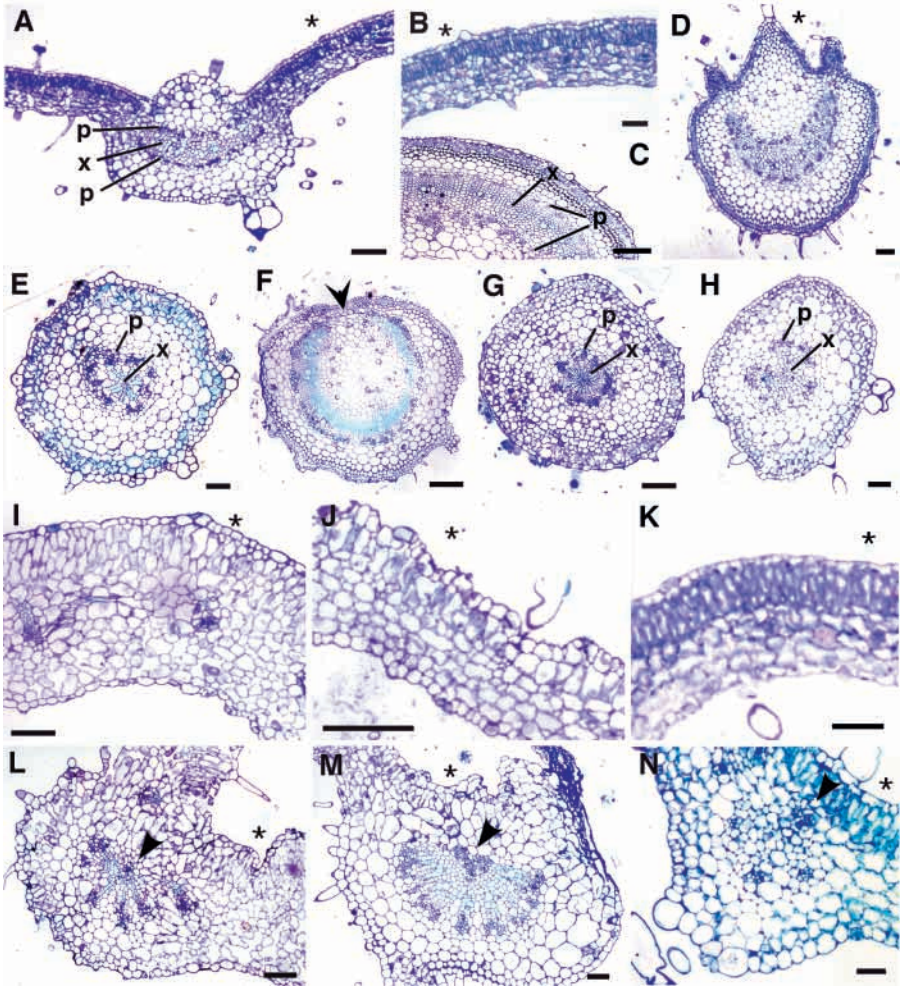

Fig. 2. Transverse sections of wild type, *w*, *w3* and *w6*. The adaxial side is marked by asterisks. (A,B) Wild-type leaf; (C) wild-type stem; (D) wild-type petiole. (E) *w3*, (G) *w6* and (H) *w* wire-like leaves. (F) *w3* leaf with ectopic distal leaves. Vascular bundle arrangement is intermediate between that of leaf and stem. (I) *w3* and (J) *w6* expanded leaves. (K) *w* expanded leaf showing normal ab-adaxiality. (L-N) Vascular bundles showing the reduced adaxial domain (arrowheads) in *w3* (L), and *w6* (M) and abnormal vascular bundle in *w* expanded leaf (N). p, phloem, x, xylem. Scale bars: (A) 100 μm, (B,E-L) 50 μm, (C) 10 μm, (D) 20 μm, (M,N) 25 μm.

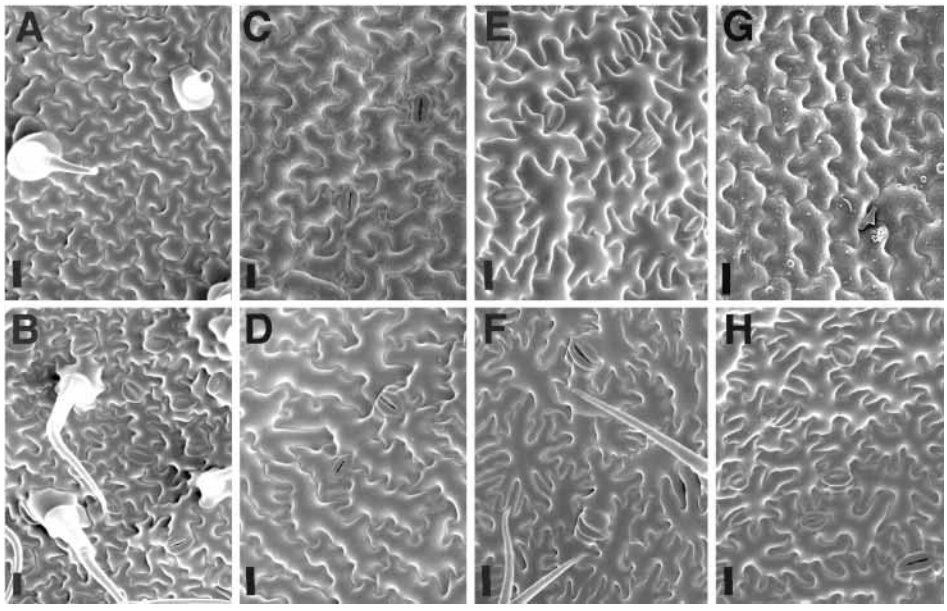

Fig. 3. Scanning electron micrographs showing epidermal cells of wild-type and wiry mutant leaves. (A,C,E,G) adaxial and (B,D,F,H) abaxial epidermis of (A,B) wild-type leaves, (C,D) *w3* leaves, (E,F) *w6* leaves and (G,H) *w* leaves. Scale bars: (A-H) 20 μm.

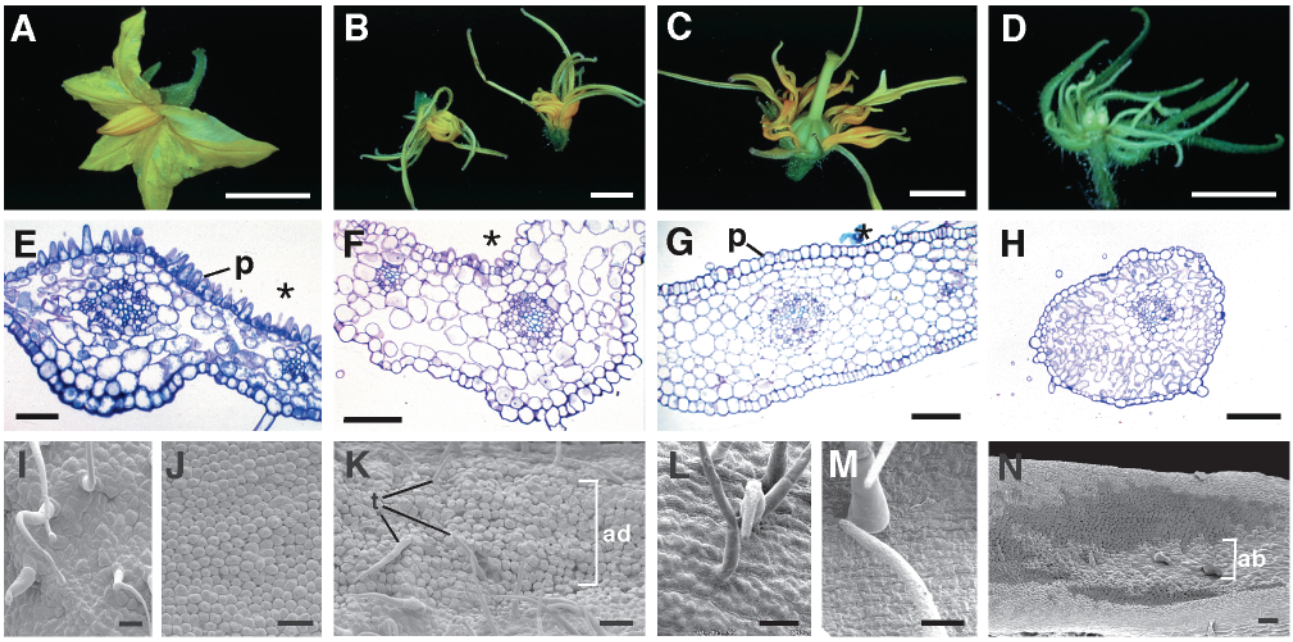

Fig. 4. Floral organ phenotypes in wild-type (A,E,I,J), *w6* (B,F,K), *w3* (C,G,L,M) and *w* (D,H,N). (A-D) Whole flowers. (E,I,J) Wild-type petal showing adaxial epidermis with papillar cells (E,J) and abaxial epidermis with flat cells and trichomes (E,I). (F-N) SEM and sections of wiry mutant petals showed partial (F,K,N) and complete (G,L,M) abaxialization of petals. (K) Trichomes (t) on adaxial (ad) petal epidermis in *w6*. SEMs of (L) abaxial and (M) adaxial petal epidermal cells in *w3*. (N) Abaxial patches (ab) were seen in adaxial petal epidermis in *w* flowers. Scale bars: (A-D) 0.5 cm, (E-N) 50 μm, (L,M) 25 μm.

seen in normal flowers. The lateral organs of *w*, *w3* and *w6* flowers were narrower than those of wild type (Fig. 4B-D).

In wild-type petals, the adaxial epidermis has protruding papillar cells and no trichomes, while the abaxial epidermis has flattened cells and many trichomes (Fig. 4E,I,J). Vascular bundle organization was not altered in the *w6* petal (Fig. 4F). However, papillar cells of the *w6* adaxial epidermis had interspersed trichomes. Moreover, the boundary between papillar cells and flattened cells was moved from the margin of the petal toward the adaxial side, indicating a reduction in the adaxial domain of the *w6* petal (Fig. 4K). In *w3* plants, petals were even more abaxialized than in *w6* petals; both the abaxial and adaxial epidermal surfaces of *w3* petal had flattened cells and numerous trichomes (Fig. 4L,M). The *w3* petal had all inner cells packed tightly, resembling the collenchyma cells of the midvein regions (Fig. 4G). Frequently, *w* petals were radially symmetric and among the papillar cells abaxial patches of flattened cells were seen (Fig. 4H,N). A summary of *wiry* mutant phenotypes is given in Table 2.

Tomato *PHANTASTICA* (*LePHAN*) expression is altered in *w*, *w3* and *w6*

phan mutant plants have a reduced adaxial domain in leaves. We determined if the abaxialized phenotypes and reduced leaflet formation in *w*, *w3* and *w6* are due to defects in *PHAN* expression. Southern blot analysis showed one copy of *LePHAN* in the tomato genome (data not shown). The chromosome location of *LePHAN* does not coincide with that of *w*, *w4* and *w6*. *LePHAN* mRNA expression was determined by conventional in situ hybridization and by RT-PCR in situ hybridization in the shoot apices and leaves of wild-type, *w*, *w3* and *w6* plants.

In the wild-type apex, *LePHAN* mRNA levels were several-fold higher in the leaf primordia than in the SAM central zone. During early leaf development in wild type, *LePHAN* transcripts were detected in both adaxial and abaxial sides of the leaf primordium (Fig. 5A), but later, as the leaf primordium grew out, *LePHAN* mRNA was confined to the adaxial side (Fig. 5B). At later developmental stages, strong *LePHAN*

Table 2. Summary of wiry mutant phenotypes

Mutant	*w*	*w3*	*w6*	*w4*
Cotyledon	Mesophyll abaxialized	Mesophyll abaxialized	Vascular tissue abaxialized	Completely abaxialized
Radially symmetric leaves	Abaxialized	Abaxialized with ectopic buds	Abaxialized	N/S
Flattened leaf	Normal	Abaxialized	Abaxialized	N/S
Axillary buds	Normal	Normal	Normal	N/S
Flower organs	Abaxialized	Abaxialized	Abaxialized	N/S
Flower organ numbers	Increased	Increased	Increased	N/S
LePHAN expression	Reduced	Reduced	Reduced	N/S
LeYAB B expression	No expression	On the adaxial side	On both ab-adaxial sides	N/S
LeT6 expression	Reduced	Reduced	Reduced	N/S
TKN1 expression	Reduced	Reduced	Increased	N/S

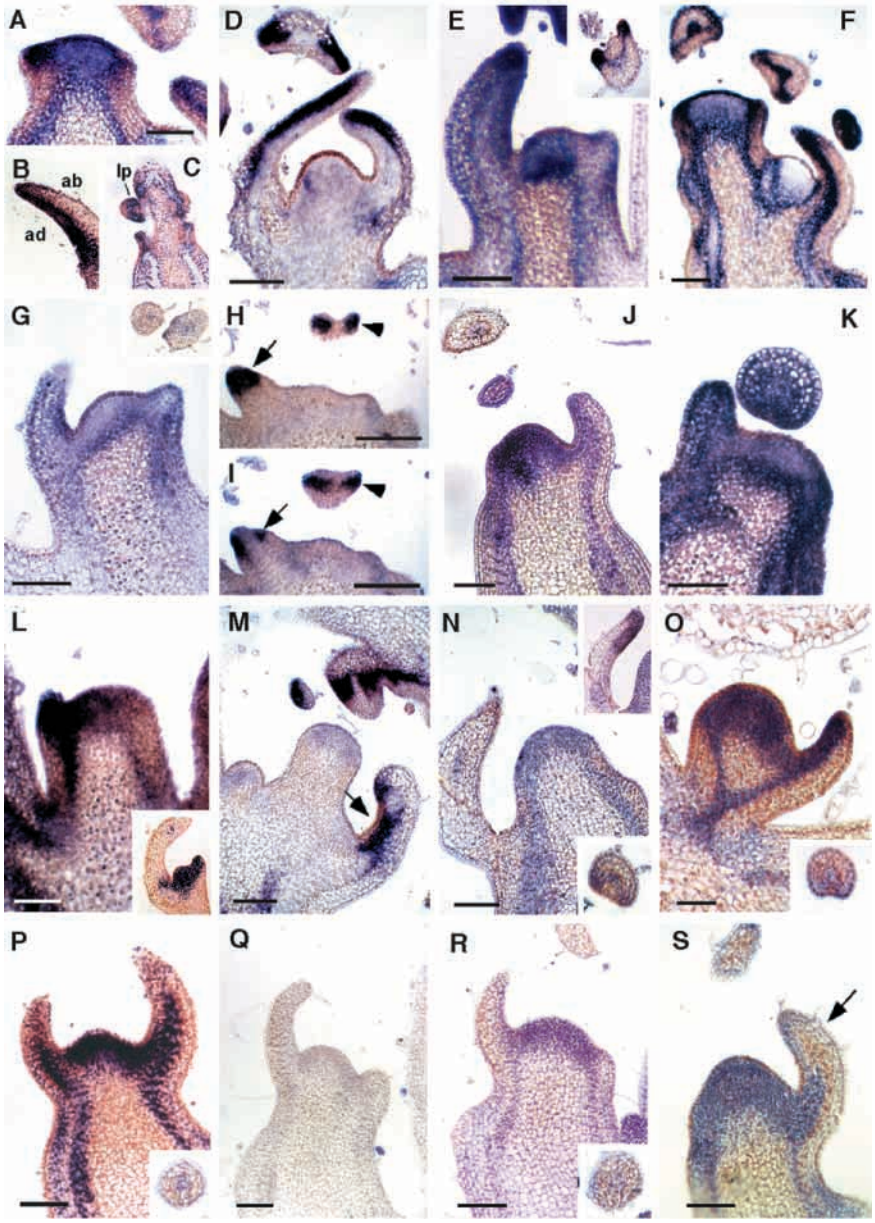

Fig. 5. In situ hybridization showing *LePHAN*, *LeYAB B* and *KNOX* expression patterns in the SAM. (A-F) Wild type, (G-K) *w6*, (L-O) *w3* and (P-S) *w*. mRNA accumulation patterns for *LePHAN* (A,B,C,G,L,P), *LeYAB B* (D,H,I,M,Q), *LeT6* (E,J,N,R) and *TKN1* (F,K,O,S). (A-C) *LePHAN* expression in the wild-type SAM and young leaf primordium (A), later stage leaf primordium (B) and leaflet primordia (lp; C). (G,L,P) *LePHAN* expression in the SAM and young leaf primordium of *w6* (G) *w3* (L) and *w* (P). Downregulation of *LePHAN* was seen in later leaf primordia in *w6* (G inset), *w3* (L inset) and *w* (P inset). (D) *LeYAB B* expression in the wild-type leaf primordium. (H,I,M,Q) *YAB B* was expressed in both adaxial and abaxial domains of leaf primordium in *w6* (H,I), but only in adaxial domain in *w3* (M), and no *YAB B* expression was detected in *w* leaf primordium (Q). (E) *LeT6* expression in the wild-type SAM, leaf primordium and leaflet primordium (E inset). (J) *LeT6* expression in the SAM and young leaf primordium in *w6*, and in later leaf primordia (J inset). (N,R) *LeT6* expression in the SAM and the later stages of *w3* (N, N inset) and *w* (R,R inset) leaf primordium. (F,K,O,S) *TKN1* expression in wild type (F), *w6* (K), *w3* (O) and *w* (S). *TKN1* expression in later stages of leaf primordium of *w3* (O inset). Scale bars: 50 μm.

expression was detected in the leaflet primordium and immature leaflet lamina regions (Fig. 5C).

In the *w6* SAM, reduced *LePHAN* expression was detected in the central zone. Moreover, *LePHAN* expression in the early leaf primordium, especially on the adaxial side, was much reduced compared to that in wild type (Fig. 5G). No *LePHAN* was detected in later stages of leaf development of wire-like leaves, but the *LePHAN* transcript was detected in growing leaflet primordia and leaflet blades in the developing cup-shaped leaves and in leaves with reduced leaflet numbers (Fig. 5G inset). In *w* and *w3* plants, no alteration of *LePHAN* mRNA accumulation was detected in the SAM and early stage leaf primordia, but *LePHAN* expression was absent in the later stages of wire-like leaves (Fig. 5L,P and insets).

LeYABBY B expression in *w*, *w3* and *w6*

To further characterize ab-adaxiality in *w*, *w3* and *w6* leaves, we examined the expression of *LeYAB B*, a member of the *YABBY* gene family, in leaves of wild-type and the wiry mutants. *LeYAB B* was expressed in the abaxial regions of wild-type leaf primordia (Fig. 5D). In *w6* plants, *LeYAB B* expression was seen in both the adaxial and abaxial sides of later radial leaves, and serial sections showed a hollow tube-like pattern of expression (Fig. 5H,I arrow). Earlier flattened *w6* leaves showed a wild-type *LeYAB B* expression pattern (Fig. 5H,I arrowheads). Interestingly, in the *w3* leaf primordium, *LeYAB B* mRNA accumulated on the adaxial instead of the abaxial side (Fig. 5M arrow). No *LeYAB B* expression was detected in the *w* leaf primordium (Fig. 5Q). These results were confirmed by northern hybridization to RNA extracted from *wiry* shoot apices (data not shown).

Expression of *LeT6* and *TKN1* in *w*, *w3* and *w6*

To determine if reduced leaflet formation in *w3* and *w6* leaves

is due to the alteration of class I *KNOX* gene expression and to determine the regulatory relationship between *LePHAN* and *KNOX* genes in tomato, the mRNA expression patterns of two class I *KNOX* genes, *LeT6* (the tomato *STM* ortholog) and *TKN1* (the tomato *KNAT1* ortholog) were examined.

In wild type, *LeT6* mRNA accumulates in the SAM, in the early leaf primordia, and later in leaflet primordia and growing leaflet blades (Fig. 5E) (Chen et al., 1997). Strong *LeT6* expression was detected in the central zone of wild-type SAM (Fig. 5E). In the *w* and *w3* mutants less *LeT6* mRNA was detected in the region of the SAM (Fig. 5N,R). Downregulation of *LeT6* mRNA was seen in later stages of *w*, *w3* and *w6* leaf development. This is equivalent to the stage producing leaflet primordia and growing leaflet lamina in wild type. No *LeT6* mRNA could be seen in *w6* plants that were producing wire-like leaves (Fig. 5J inset, R inset). However, *LeT6* mRNA localized in the leaflet and leaflet lamina regions of *w*, *w3* and *w6* plants that were producing leaves that either were cup-shaped or had a reduced number of leaflets (Fig. 5N insets).

TKN1 expression could be seen in the wild-type SAM, leaf primordia and growing leaflet lamina, but the signal was stronger in the leaf primordia and the peripheral zone of the SAM than in the central zone (Fig. 5F). In *w6* plants, a high level of *TKN1* RNA was detected throughout the SAM and in both early and late leaf primordia, including the radially symmetrical primordia (Fig. 5K). In *w3* and *w*, *TKN1* expression was normal in the SAMs but downregulated in the leaflet primordia (Fig. 5O,S). Expression of *TKN1* was absent at the tip of the leaf primordium (distal region), where abaxialized wire-like structures are seen in *w* shoots (Fig. 5S arrow).

The expressions of *KNOX* genes (*LeT6* and *TKN1*) were altered in *wiry* mutants. In particular, downregulation of *LeT6* in later stage of leaf primordia was accompanied by reduction of leaf compounding in *wiry* mutants. Reduction of *LePHAN* expression and upregulation of *TKN1* in *w6* suggests a negative regulatory relationship between *LePHAN* and *TKN1*.

LeT6 is a negative regulator of *LePHAN* in tomato

To determine if *LeT6* regulates *LePHAN* in tomato, we analyzed *LePHAN* expression in *Curl* (*Cu*), a mutant known to overexpress *LeT6* (Parnis et al., 1997). As reported (Parnis et al., 1997), ectopic expression of the *LeT6* mRNA was detected in *Cu* leaflets and leaflet lamina (Fig. 6A). In *Cu* plants, *LePHAN* was present but reduced in the leaf primordia, leaflet and leaflet blade regions (Fig. 6B,C). This *LePHAN* downregulation in *Cu* was not sufficient to cause a *LePHAN* downregulation phenotype, as the *Cu* leaf showed normal anatomy and epidermal cells (data not shown). Another *LeT6* overexpression mutation, *Mouse Ears* (*Me*), is caused by a gene duplication that leads to early overexpression of a homeobox-containing fusion RNA (Chen et al., 1997; Janssen et al., 1998; Parnis et al., 1997). In the *Me* mutant, *LePHAN* expression was reduced and the location of expression was altered (Fig. 6E,F). In the *Me* plants, *LePHAN* expression was reduced in the proximal region of the leaf primordia (data not shown) and confined to a narrower adaxial domain in leaf primordia (Fig. 6E). Often, LePHAN expression was absent from the leaf primordia of *Me/Me* (Fig. 6F), except in vascular tissues, and the leaves produced were radial. This downregulation of *LePHAN* correlated with the production simple leaves and wire-like leaves at the upper nodes in *Me/Me* plants (Fig. 6O), phenocopying *LePHAN* downregulation phenotypes (Fig. 6J). In these *LePHAN* antisense transgenic plants, LePHAN expression was reduced to a narrow domain or only to vascular tissues (Kim et al., 2003), similar to *LePHAN* expression in *Me/Me* (Fig. 6F). Together, these data suggest that *LeT6* is a negative regulator of *LePHAN*.

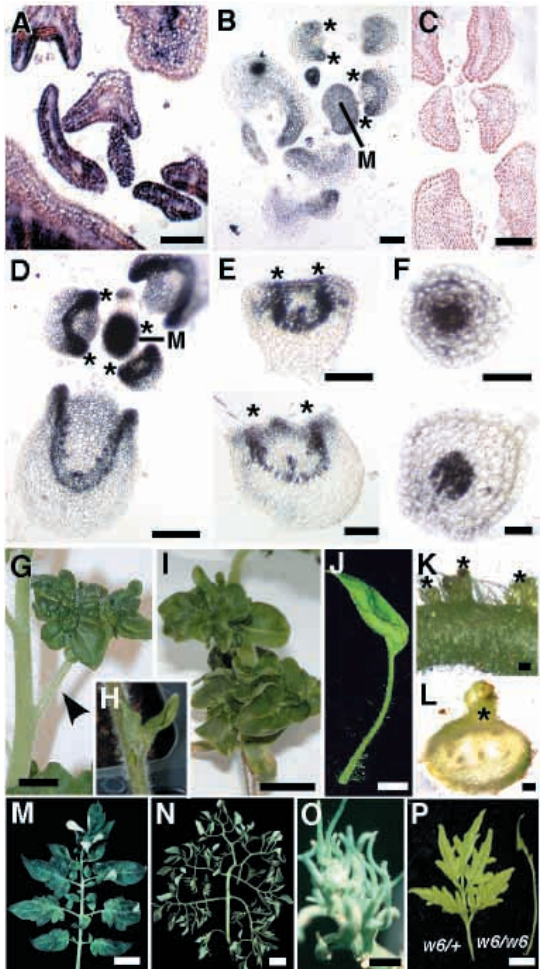

Fig. 6. Genetic interaction between *KNOX* genes and *LePHAN*. (A) Overexpression of *LeT6* in *Cu* leaflets. (B,C) *LePHAN* expression in the *Cu* leaf primordia and (C) leaflet primordia. (D-F) LePHAN accumulation in the wild-type and *Me* plants. (D) A transverse section of a wild-type SAM. (E,F) Transverse sections of (E) *Me* leaf primordium with narrow adaxial domain, and (F) radial *Me* leaf. (B,D,E) Asterisks in B,D and E indicate the LePHAN expression domains; M, meristem. (G,H) Reduced *Cu* phenotypes in *antiLePHAN* background. Arrowhead points to a radially symmetric expanded petiole. (I) *Cu* plants showing *LeT6* overexpression phenotypes. (J) *LePHAN* downregulation phenotype in the *antiLePHAN* leaf. (K,L) Ectopic shoots (*) on adaxial domains in *Me* leaf. (M-P) *Me* and *w6* phenotypes are dosage sensitive. (M) Unipinnate wild-type (+/+) tomato leaf. (N) Excessively compound leaf in *Me/+*. (O) Wire-like leaves in *Me/Me*. (P) *w6/+* leaf is more lobed than normal leaves and *w6/w6* leaves are wire-like or cup-shaped. Scale bars: (A-F) 50 μm, (K, L) 100 μm, (J) 0.5 cm and (G-I, M-P) 1 cm.

LeT6 requires *LePHAN* activity in leaf primordium

To determine if *LeT6* expression is in turn regulated by *LePHAN*, we made use of a *LePHAN* antisense transgenic line. Several independent *LePHAN* antisense transgenic lines showed cup shaped or wire-like leaves (Fig. 6J) and immunolocalization and in situ RT-PCR experiments showed that *LePHAN* levels were reduced in these plants and petioles were radial (Kim et al., 2003).

When *Cu* was crossed into a *LePHAN* antisense transgenic line, the *Cu* phenotype was less severe, having less curled leaves and often cup-shaped leaves with simple leaf blades (Fig. 6H). The curled leaf phenotypes were confined to distal region of the leaf. These plants showed elongated and radially symmetric petioles (Fig. 6G). These results suggest that *LePHAN* downregulation phenotype is epistatic to *Cu* and that the *LeT6* overexpression phenotypes of *Cu* require *LePHAN* activity. In *Me/Me*, *LeT6* overexpression also led to the production of ectopic shoots on the leaves (Fig. 6K,L, asterisk). These ectopic shoots were formed only in the narrow adaxial domains, where *LePHAN* was expressed (Fig. 6E). Often this adaxial domain converged to a point (Fig. 6L) and ectopic shoots emerged at this point, suggesting that *LePHAN* activity is required for *LeT6* overexpression phenotypes in *Me.*

Because a phenocopy of *PHAN* downregulation is seen only in homozygous *Me*, but not in heterozygous *Me* plants, the suppression of *LePHAN* by *LeT6* seems to be dosage sensitive. *Me/+* plants show a typical *KNOX* overexpression phenotype with an increase in leaf compounding (Chen et al., 1997; Parnis et al., 1997) (Fig. 6N). Similarly, *w6/w6* homozygous plants (with reduced *LePHAN* levels) generated wire-like leaves (Fig. 6P) while, *w6/+* heterozygous plants produced lobed leaves, a phenotype also seen in the plants overexpressing *KNAT1* in *Arabidopsis* (Fig. 6P).

DISCUSSION

Abaxialization of *w*, *w3* and *w6* leaves leads to reduced leaflet formation

In *w*, *w3* and *w6* mutant plants, partial or complete abaxialization of the lateral organs was observed throughout development, suggesting that *W*, *W3* and *W6* play important roles in establishing adaxial cell identity in all lateral organs. The tomato leaf primordium develops basipetally (Dengler, 1984) and preferential distal abaxialization in the *w* leaves indicates that *W* acts during early leaf development (in the distal region), whereas *W3* and *W6* function later (in the proximal region) in leaf development. Abaxialization of the wire-like leaf in *w*, *w3* and *w6* is different from proximalization of the distal region (changing blade domain into sheath domain) seen in maize mutants (Becraft, 1994; Sinha and Hake, 1994; Tsiantis et al., 1999) as unlike petioles, wiry leaves are radially symmetrical. However, the occasional production of stem-like leaves that have ectopic leaves with ectopic axillary buds (in *w3*) suggests that proximalization into stem-like identity can occur in addition to abaxialization. Adaxial cells are thought to be necessary for the induction of axillary buds in *Arabidopsis* (McConnell and Barton, 1998; McConnell et al., 2001). The abaxialized wire-like leaves of *w*, *w3* and *w6* formed normal axillary buds on the adaxial side of the leaf base (Fig. 1F). Sessile (*Arabidopsis*) and petiolated (tomato) leaves may have different potentials to form axillary buds in their leaf bases, and adaxial cell fate may not be an absolute requirement for axillary bud formation in tomato. This could also account for the presence of ectopic axillary buds in *w3* mutant leaves.

It has been proposed that the boundary between abaxial and adaxial cell fates is important for lateral lamina outgrowth (Bowman et al., 2002; Lynn et al., 1999; McConnell and Barton, 1998; Timmermans et al., 1998). Reduced adaxial domain is accompanied by significantly reduced leaflet numbers in *w, w3* and *w6* (Table 1). One explanation for the fewer leaflets in *w*, *w3* and *w6* compound leaves is that leaflet primordium formation, like lamina outgrowth, also requires a proper ab-adaxial boundary.

LePHAN expression in *w*, *w3* and *w6*

Two aspects of *LePHAN* expression in tomato set it apart from orthologs in other species. No other *PHAN* ortholog has been reported to be expressed in the SAM or specifically in the adaxial domain of leaf primordia. At later stages of leaf development, *LePHAN* is expressed only in the region of leaflet primordium initiation (Fig. 5C), suggesting that *LePHAN* (like *LeT6*) might be involved in leaflet formation, or in establishing ab-adaxiality of leaflets. The possible function of *LePHAN* in leaflet development is also supported by the fact that *LePHAN* is not expressed in wire-like leaves and localizes to the growing leaflet primordium or leaflet lamina region in cup-shaped, or less compound leaves of *w*, *w3* and *w6.* Downregulation of *LePHAN* was seen in the leaf primordium and leaflet primordium in these mutants, suggesting that *W*, *W3* and *W6* are positive regulators of *LePHAN* expression in leaves. In addition, *W6* may also regulate *LePHAN* expression positively in the meristem.

Regulatory relationship between *LePHAN* and *KNOX* genes in tomato

Tomato *LePHAN* expression was reported to be absent from the SAM in one study (Pien et al., 2001) but was seen in the SAM and leaf primordia in a domain that overlaps the *KNOX* expression domain by others (Koltai and Bird, 2000). Our results indicate that the latter is the case and that *LePHAN* (Fig. 5A, Fig. 6F,G) and *TKN1* are expressed most strongly in the peripheral zone of the meristem, whereas *LeT6* expresses strongly in the central zone of the meristem (Fig. 5A,E,F). In *Arabidopsis*, *STM* is a negative regulator of *AS1*. This regulatory relationship is conserved to a large extent in tomato. In *Cu* and *Me* (*LeT6* overexpression mutants), *LePHAN* was reduced, suggesting that *LeT6* is a negative regulator of *LePHAN*. *TKN1* was upregulated in *w6* where *LePHAN* was downregulated. A simple interpretation for the upregulation of *TKN1* in *w6* is that *LePHAN* is a negative regulator of *TKN1*. However, it is unclear how *LePHAN* and *TKN1* express in an overlapping manner in both the SAM and early leaf primordia. Perhaps *LePHAN* and another gene (gene A) have a mutually exclusive relationship and gene A in turn inhibits *TKN1* expression.

The regulatory dynamics between *LePHAN*, *TKN1* and *LeT6* in later leaf and leaflet primordia is different from that in the meristem and early leaf primordium. *LePHAN*, *TKN1* and *LeT6* all express in the leaflet primordium and all of them are downregulated in the wire-like leaves of *w* and *w3*. These

expression data imply that the negative regulation of *LeT6* on *LePHAN* seen in the meristem region does not hold in the wild-type leaflet primordium. Rather, *LePHAN* functions with *LeT6* in a coordinate manner. *Cu* phenotypes were reduced in anti*LePHAN/+* plants and *Cu* and *Me* phenotypes were confined to the region where *LePHAN* was expressed, suggesting that the *LeT6* overexpression phenotype requires *LePHAN* function. Similarly, downregulation of *LePHAN* masked *TKN1* overexpression phenotypes in *w6/w6* and suggests that *TKN1* also requires sufficient *LePHAN* activity in the leaflet primordium in tomato.

LeT6 regulation of *LePHAN* is dosage sensitive

A reduced blade phenotype can be seen only in homozygous *Me/Me* plants (Fig. 6O) and not in heterozygous *Me/+* plants (Fig. 6N), implying that a high dose of *LeT6* is needed to downregulate *LePHAN* in tomato. This hypothesis is also supported by the fact that the expression domains where *LePHAN* and *LeT6* express strongly do not overlap (Fig. 5A,E). We suggest that low levels of overexpression of either *LeT6* or *TKN1* in leaf primordia can cause *KNOX* overexpression phenotypes (such as increased dissection of leaves, or more lobed or heart shaped leaves with palmate venation), but high levels of *LeT6* overexpression might lead to severe *LePHAN* downregulation, causing a *LePHAN* downregulation phenotype. Thus, *w6/+* heterozygous plants produced highly lobed leaves (Fig. 6P), a phenotype generally attributed to *KNOX* gene overexpression, whereas *w6/w6* homozygous plants generated mostly cup-shaped or wire-like leaves, which is a *LePHAN* downregulation phenotype (Fig. 6P). Furthermore, this *LePHAN* downregulation phenotype masks the *KNOX* overexpression phenotypes in tomato, because a certain level of *LePHAN* is required for the *KNOX* overexpression phenotypes (as seen in *Cu* crossed to anti*LePHAN* and *Me/Me*). This idea is supported by some of the phenotypes seen in tomato plants that overexpress *35S::LeT6*. Some *35S::LeT6* transgenic lines showed wire-like radially symmetrical leaves, resembling *PHAN* downregulation phenotypes, instead of the typical *LeT6* overexpression phenotypes with more leaflets. *LeT6* overexpression was at much higher level in these plants producing wire-like leaves, than in plants showing leaflet overproliferation phenotypes (Janssen et al., 1998).

We propose a model (Fig. 7) that summarizes how *LeT6* and *LePHAN* are regulated in tomato. Our results suggest that *LeT6* and *LePHAN* have a mutually antagonistic expression pattern and that each is affected by the quantity of the other. Thus, high levels of *LePHAN* repress *LeT6* and similarly high levels of *LeT6* repress *LePHAN*. Our data does not support increase in *LeT6* expression by low levels of *LePHAN* and vice versa. At intermediate levels both these genes express. Since *LeT6* is thought to be necessary for meristem formation in higher plants (although this has not been directly demonstrated in tomato), loss of *LeT6* gene function or downregulation of *LeT6* could be lethal for plants. Low transformation and plant regeneration success in experiments using *35S::LePHAN* constructs support this hypothesis (our unpublished data). *LePHAN* and *LeT6* levels are well balanced in the wild-type leaf, producing 7-9 leaflets with normal ab-adaxiality. Weak *LeT6* overexpression and *LePHAN* downregulation lead to *LeT6* overexpression phenotypes seen in the 35S::*LeT6* plants (Janssen et al., 1998),

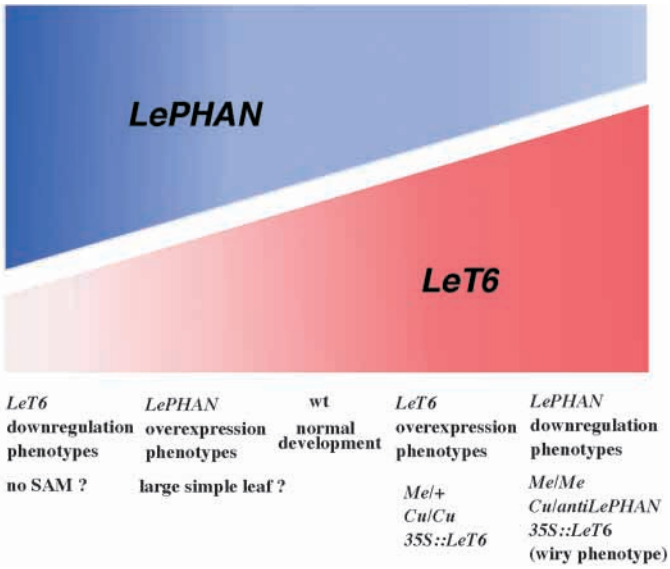

Fig. 7. A model showing the regulatory relationship between *LeT6* and *LePHAN* and final leaf morphology in tomato. *LeT6* is downregulated when *LePHAN* is strongly overexpressed. Loss of *KNOX* gene function or extreme downregulation of *LeT6* could be lethal for plants because of the lack of SAM formation/maintenance. Weak *LePHAN* overexpression might lead to the ectopic leaf blade outgrowth in the rachis region and make large simple leaves. *LePHAN* and *LeT6* levels are well balanced in the wild-type leaf, producing 8-9 leaflets with normal ab-adaxiality. Weak *LeT6* overexpression and *LePHAN* downregulation lead to *KNOX* overexpression phenotypes seen in the *35S::LeT6* plants (Janssen et al., 1998), *Me/+* and *Cu* leaves. Because *LeT6* overexpression phenotypes require *LePHAN* activity, strong *LeT6* overexpression and *LePHAN* downregulation cause *LePHAN* downregulation phenotypes including cup-shaped or wire-like leaves, severe *35S::LeT6*, *Me/Me*, *w6/w6* and *Cu/Cu;antiLePHAN/+* leaves.

Me/+ and *Cu* leaves. We suggest that the *as1* mutation showing only *KNOX* overexpression phenotypes in *Arabidopsis* and the *rs2* phenotype in maize can be categorized in this group. Perhaps, in these instances, *KNOX* overexpression does not reach a level that would cause leaf lobing or the *PHAN* downregulation phenotype. Strong *KNOX* overexpression and *LePHAN* downregulation cause *LePHAN* downregulation phenotypes including cup-shaped or wire-like leaves, as seen in the *as1* strong allele (Sun et al., 2002), severe *35S::LeT6*, *Me/Me*, *w6/w6* and *Cu/Cu;antiLePHAN/+* leaves. However, it should be emphasized that a direct interaction between the *KNOX* genes and *PHAN* has not been proved and this interaction may involve multiple regulatory steps.

LeYAB B expression in *w, w3* and *w6* meristems

Our results show that, as seen for the *Arabidopsis YAB3* gene, *LeYAB B* is a good marker for abaxial cell fates (Fig. 5D). *LeYAB B* mRNA was detected throughout *w6* leaf primordia (Fig. 6H, I), while *LePHAN* mRNA was downregulated in the adaxial region of the leaf (Fig. 5G). This suggests that adaxial cells of leaf primordia in *w6* are converted into abaxial cells. These results are consistent with the complete abaxialization of adaxial cells of the *w6* leaf (Fig. 2G). However, *LeYAB B* was unable to downregulate *LePHAN* in the adaxial region of *w3* leaf primordium (Fig. 5L,M) and the absence of *LeYAB B*

did not cause ectopic expression of *LePHAN* in *w* (Fig. 5P,Q). In *w3*, *LeYAB B* was expressed in the adaxial region of the lateral organs (Fig. 5M). However, in *w3*, the absence of *LeYAB B* in the abaxial region still allowed cells to have abaxial fate. While, presence of *LeYAB B* in the adaxial domain did not cause complete abaxialization, the adaxial epidermis attained some abaxial features (Fig. 3C), suggesting that *LeYAB B* may play a role in the acquisition of abaxial cell fates. *yab* and *fil* mutants have been reported to upregulate *KNOX* gene expression and result in ectopic shoots in *Arabidopsis* (Kumaran et al., 2002). By contrast in tomato, ectopic expression of *LeYAB B* in the adaxial region and absence in the abaxial region of the *w3* leaf accompanies ectopic bud formation in these leaves. The fact that ectopic expression of *LeYAB B* in the adaxial region was detected in both *w3* and *w6* leaf primordia, but *LePHAN* was downregulated only in *w6* leaf primordium, and complete abaxialization of adaxial cells was seen only in *w6* leaf all suggest that *LePHAN* and other adaxial specific genes play a major role in controlling ab-adaxiality, while the *YABBY* genes might be involved in a downstream part of the cell fate acquisition pathway. Our results suggest that *KNOX* gene expression is regulated by presence or absence of *LePHAN* and not *LeYAB B* in tomato.

Is a compound leaf a reiterated shoot system or a carved simple leaf?

The origins and homologies of compound leaves have been a matter of debate. One view is that dicot compound leaves are a homeotic reiteration of simple leaves along the rachis region of a compound leaf (Lacroix and Sattler, 1994; Rutishauser, 1995). In contrast, others proposed that a compound leaf is formed by dissecting or carving a simple leaf, perhaps by inhibition of blade formation in the rachis area (Hagemann, 1984; Kaplan, 1975).

The adaxial domain is necessary for leaflet primordium formation in tomato. This is reminiscent of the situation where the adaxial domain of a leaf primordium is required for normal SAM activity, and is suggestive of some similarity between compound leaves and shoot systems. This similarity is further supported by the presence of *KNOX* gene expression in the leaflet primordia in all compound-leafed species from ferns and cycads to higher plants (Bharathan et al., 2002).

If the expression of *KNOX* genes is crucial to make compound leaves, introducing the expression of *KNOX* genes into the leaf would have been an important evolutionary innovation that led to the occurrence of compound leaves. In *Arabidopsis*, *Antirrhinum* and maize (simple leaves), no *KNOX* gene expression can be seen in the leaf primordia at any stage of leaf development. Perhaps this is due to the fact that *PHAN* and *KNOX* have a very tight mutually exclusive regulatory relationship in *Arabidopsis*, *Antirrhinum* and maize (Byrne et al., 2000; Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Waites et al., 1998). Our study shows that both *KNOX* (*LeT6* and *TKN1*) and *LePHAN* are expressed in leaflet primordia, suggesting that *KNOX* genes and *LePHAN* are not mutually exclusive in the tomato leaflet primordium and that their functions might be dependent on each other. Acquisition of a positive regulatory relationship between *KNOX* genes and *LePHAN* in the leaf primordium might be an evolutionarily significant change to introduce leaflet formation in the ancestral simple leaf primordium. In fact, the discovery that the regulation between *KNOX* genes and *LePHAN* of tomato differs from that of simple-leafed species raises several questions. It will be interesting to determine if the positive regulatory relationship between *KNOX* genes and *PHAN* is conserved among compound-leafed species and if this positive regulation is responsible for allowing *KNOX* expression in leaf primordia of compound-leafed species.

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