Specification of adaxial cell fate during maize leaf development

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Summary

Dorsoventral (adaxial/abaxial) polarity of the maize leaf is established in the meristem and is maintained throughout organ development to coordinate proper outgrowth and patterning of the leaf. *rolled leaf1* (*rld1*) and *leafbladeless1* (*lbl1*) are required for the specification of the adaxial/upper leaf surface. *rld1* encodes a class III homeodomain-leucine zipper (HD-ZIPIII) protein whose adaxial expression is spatially defined by miRNA166-directed transcript cleavage on the abaxial side. The semi-dominant *Rld1-Original* (*Rld1-O*) mutation, which results from a single nucleotide substitution in the miRNA166 complementary site, leads to persistent expression of mutant transcripts on the abaxial site. This causes the adaxialization or partial reversal of leaf polarity. By contrast, recessive mutations in *lbl1* cause the formation of abaxialized leaves. The *lbl1* and *Rld1-O* mutations mutually suppress each other, indicating that these two genes act in the same genetic pathway. Adaxial and meristematic expression of *rld1* is reduced in *lbl1* mutants, indicating that *lbl1* acts upstream of *rld1* to specify adaxial fate during primordium development. However, *rld1* expression in the vasculature of *lbl1* is normal, suggesting that the specification of adaxial/abaxial polarity during vascular and primordia development is governed by separate but overlapping pathways. We also show that members of the maize *yabby* gene family are expressed on the adaxial side of incipient and developing leaf primordia. This expression pattern is unlike that observed in *Arabidopsis*, where *YABBY* expression is correlated with abaxial cell fate. The *yabby* expression patterns in *lbl1* and *Rld1-O* mutants suggest that the *yabby* genes act downstream in the same pathway as *lbl1* and *rld1*. Moreover, our observations suggest that maize *yabby* genes may direct lateral organ outgrowth rather than determine cell fate. We propose that a single genetic pathway involving *lbl1, rld1* and the *yabby* genes integrates positional information within the SAM, and leads to adaxial/abaxial patterning and mediolateral outgrowth of the leaf.

Key words: Maize, leaf, Dorsoventral, Adaxial, Abaxial, Meristem, miRNA, *leafbladeless1*, *Rolled leaf1*, YABBY

Introduction

Leaves of higher plants exhibit a varying degree of asymmetry along the adaxial/abaxial axis. This asymmetry is thought to reflect inherent positional differences in the developing organ relative to the shoot apical meristem (SAM) from which it arises (Wardlaw, 1949). Specification of adaxial cell fate may indeed require a meristem-borne signal as separation of incipient primordia from the SAM by incision results in the formation of radially symmetric abaxialized leaves (Sussex, 1951; Sussex, 1955; Snow and Snow, 1959; Hanawa, 1961). Members of the class III homeodomain-leucine zipper (HD-ZIPIII) family, which includes the maize ROLLED LEAF1 (RLD1), and the *Arabidopsis* PHABULOSA (PHB), PHAVOLUTA (PHV) and REVOLUTA (REV) proteins, are required to establish adaxial identity in lateral organ primordia (McConnell et al., 2001; Emery et al., 2003; Juarez et al., 2004). These proteins contain a START lipid-sterol binding-like domain and may specify adaxial cell fate by conveying the hypothetical meristem-borne signal (McConnell et al., 2001; Eshed et al., 2001; Kidner et al., 2002).

rld1 and the *Arabidopsis HD-ZIPIII* genes are expressed in the central region of the SAM and throughout incipient leaf primordia. Upon primordium emergence, *HD-ZIPIII* expression becomes restricted to the vasculature and the adaxial side (McConnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003; Juarez et al., 2004). In both maize and *Arabidopsis*, this polar expression pattern is set up by the abaxial expression of miRNA166 or miRNA165 (Juarez et al., 2004; Kidner and Martienssen, 2004), which show extensive complementarity to *HD-ZIPIII* transcripts and direct their cleavage (Reinhart et al., 2002; Rhoades et al., 2002; Tang et al., 2003). Single nucleotide substitutions that disrupt the *rld1* miRNA166 complementary site, as in the semi-dominant mutant *Rld1-Original* (*Rld1-O*), lead to the persistent expression of *rld1* transcripts on the abaxial side of leaf primordia (Juarez et al., 2004). As a result, *Rld1-O* leaves become adaxialized or partially reverse leaf polarity (Nelson et al., 2002). Similarly, mutations in the miRNA165/166 complementary site of *PHB*, *PHV* and *REV* are dominant and cause adaxial/abaxial patterning defects (McConnell et al., 1998; McConnell et al., 2001; Emery et al., 2003).

Establishment of abaxial identity in *Arabidopsis* requires the *KANADI* and *YABBY* genes, in addition to miRNA165 and miRNA166. *KAN1* and *KAN2* encode redundant transcriptional regulators belonging to the GARP family (Eshed et al., 2001; Kerstetter et al., 2001). *KAN1* is expressed throughout young

organ primordia but becomes abaxially localized shortly after *PHB* transcripts become restricted to the adaxial domain. Consistent with this expression pattern, lateral organs of *kan1 kan2* mutants are narrow or radial, and adaxialized. The *YABBY* gene family comprises six members, including the vegetatively expressed *FILAMENTOUS FLOWER* (*FIL*), *YAB2* and *YAB3* (Sawa et al., 1999; Siegfried et al., 1999). YABBY proteins contain a zinc finger and a helix-loop-helix domain (YABBY domain), and may also function as transcriptional regulators. *FIL*, *YAB2* and *YAB3* are initially expressed throughout incipient primordia but become restricted to the abaxial side of all developing organs. These expression patterns are altered in the *phb1-d* and *kan1 kan2* double mutants, suggesting that the *YABBY* genes act after adaxial/abaxial polarity is established (Siegfried et al., 1999; Eshed et al., 2001). *fil* and *yab3* have redundant functions but, in combination, *fil yab3* cause a partial adaxialization of the leaf (Siegfried et al., 1999; Kumaran et al., 2002).

Specification of adaxial/abaxial polarity leads to the differentiation of distinct cell types within the upper and lower domains of the developing primordium, and this is also reflected in the patterning of the vasculature. Xylem tissue differentiates towards the adaxial side, whereas phloem forms on the abaxial side. Mutations in the *HD-ZIPIII* genes and *kan1 kan2* affect vascular patterning in both the leaf and the stem of the plant (McConnel and Barton, 1998; Zhong and Ye, 1999; Ratcliffe et al., 2000; Emery et al., 2003). Moreover, miRNA166 and the *hd-zipIII* genes *rld1* and *phb* are expressed in complementary domains in the vasculature, suggesting that adaxial/abaxial patterning during vascular and lateral organ development may be governed by a similar mechanism (Juarez et al., 2004). Analysis of the *Antirrhinum phantastica* (*phan*) mutant further indicated that the juxtaposition of adaxial and abaxial domains within the leaf directs mediolateral lamina outgrowth (Waites and Hudson, 1995). When this boundary is lost, as in the surgical experiments or as a result of mutation of the adaxial or abaxial determinants, radial organs are produced. By contrast, formation of additional adaxial/abaxial boundaries, as in weakly affected *phan* leaves that develop patches of abaxial cells on the adaxial leaf surface, induces the formation of ectopic lamina outgrowths.

Specification of adaxial cell fate in maize also requires normal *leafbladeless1* (*lbl1*) activity. Recessive mutations in *lbl1* lead to the formation of radially symmetric abaxialized leaves and leaf-like lateral organs (Timmermans et al., 1998). Like the weak *phan* leaves, less severe *lbl1* leaves develop patches of abaxial cells on the adaxial leaf surface, which result in bifurcation of the leaf or in the formation of fully differentiated lamina at the ectopic abaxial/adaxial boundaries. Here, we show that *lbl1* and *Rld1-0* mutually suppress each other, and that *lbl1* is required for normal *rld1* expression in the SAM and on the adaxial side of leaf primordia. *lbl1* thus acts upstream of *rld1* during the specification of adaxial cell fate in the primordium. The *rld1* expression pattern in the vasculature was unaffected in *lbl1* mutants, suggesting that adaxial/abaxial polarity in veins may be established independently of *lbl1* function. We also cloned maize homologs of the *Arabidopsis FIL* and *YAB3* genes, and show that these maize *yabby* genes, in contrast to those of *Arabidopsis*, are expressed on the adaxial side of developing leaf primordia. The expression patterns of two *yabby* genes in *lbl1* and *Rld1-O* mutants suggest they act downstream of *lbl1* and *rld1*, and may direct lateral organ outgrowth. These observations suggest that *lbl1*, *rld1* and the *yabby* genes act in the same genetic pathway leading to adaxial cell fate and mediolateral outgrowth during maize leaf development.

Materials and methods

Genetic analysis

The *Rld1-O* mutant was obtained from M. Freeling (University of California at Berkeley, Berkeley, CA) and like *lbl1-ref* and *lbl1-rgd1* introgressed three to six times into diverse inbred backgrounds. Both the *lbl1-ref* and *lbl1-rgd1* alleles are linked to the endosperm marker *white endosperm* (*y1*), which is dosage-sensitive and thus allows the identification of heterozygous and homozygous mutant individuals based on seed color. Double mutants homozygous for *lbl1* and heterozygous for *Rld1-O* were generated in the B73 inbred line by backcrossing *lbl1-ref y1*/++; *Rld1-O*/+ or *lbl1-rgd1 y1*/++; *Rld1-O*/+ double heterozygous plants to heterozygous *lbl1-ref y1*/++ and *lbl1-rgd1 y1*/++ siblings, respectively. Several hundred white (*y1y1*/*y1*) and dark yellow (++/+) progeny from these crosses were grown in the field and greenhouse. As expected, dark yellow seed segregated ~1:1 for wild-type and *Rld1-O* mutant plants, whereas the white seed segregated for *lbl1* single and *lbl1 Rld1-O* double mutants. Plant genotypes were confirmed using RFLP linkage analysis. Flanking probes *npi235* and *umc85*, and probe *umc94* were used to follow inheritance of *lbl1* and *Rld1-O*, respectively.

Scanning electron microscopy

Three to five independent mature adult leaves (leaf 9 or 10) were analyzed for wild type and each single and double mutant. Tissue samples were collected approximately midway along the length of the blade near the midvein, in the middle of the leaf lamina and at the leaf margin. Samples were fixed overnight at 4°C in 0.1 M phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde, dehydrated through an ethanol series and critical point dried. Each sample was divided into two halves prior to mounting to allow analysis of both the adaxial and abaxial epidermal surfaces. Specimens were coated with gold and analyzed on a Hitachi S-3500N SEM using an accelerating voltage of 15 kV.

Isolation of *Zea mays yabby* (*zyb*) genes

Degenerate primers, YAB5′ (TGCTAYGTSMAMTGCARCT-WYTGC) and YAB3′ (RTTYTTNGCWGCAGYRCTRAAKGC), were designed based on sequence conservation in the amino-terminal Zn-finger and carboxy-terminal YABBY domains of *FIL*, *YAB2* and *YAB3*. These primers were used at a final concentration of 2 μM and an annealing temperature of 57°C to amplify partial genomic fragments of two maize *yabby* genes. Both genomic fragments were used to screen a vegetative apex cDNA library using standard protocols. Map positions for the *zyb* genes were determined using two recombinant inbred populations (Burr et al., 1988). ClustalW alignments of the Zn-finger and YABBY domains of the *Arabidopsis* and maize YABBY proteins were generated using MacVector6.5.1 (Oxford Molecular Group), with a gap weight of 15.00 and a length weight of 0.30. Parsimony analyses were performed using PAUP4.0. A consensus tree and bootstrap values were determined after 1000 replicates.

Molecular biology

Genomic DNA and Southern blots were prepared and hybridized as described (Timmermans et al., 1996). For RT-PCR, total RNA was isolated from the apices and young leaf primordia of two-week-old seedlings using Trizol reagent (GibcoBRL). Approximately 1 ug of DNaseI-treated RNA was primed with oligo(dT) and converted to complementary DNA using M-MuLV reverse transcriptase (NEB).

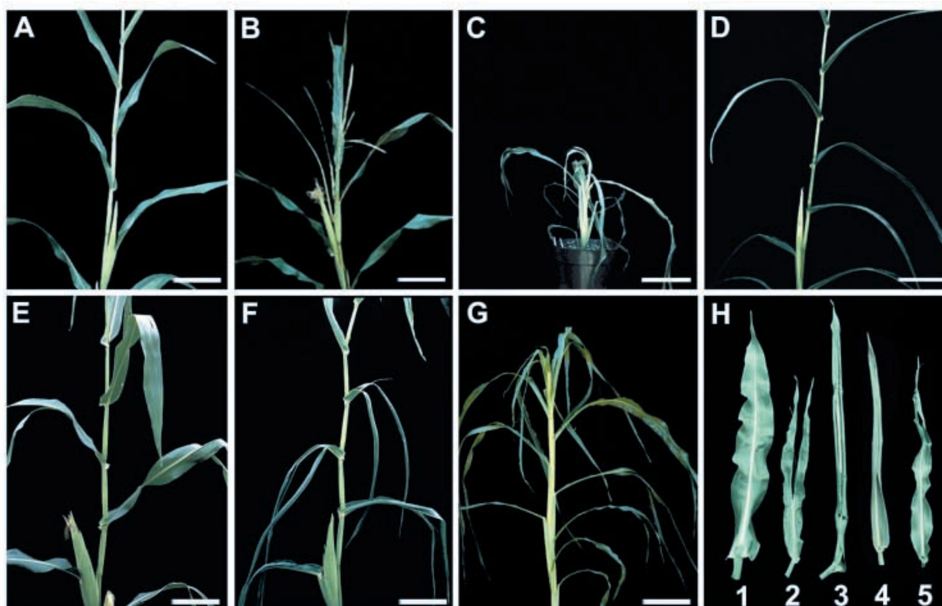

Fig. 1. *lbl1* and *Rld1-O* mutually suppress each other. Relative to wild-type B73 (A), weak *lbl1-ref* (B) and severe *lbl1-rgd1* (C) mutants have a reduced stature and develop a variety of leaf phenotypes with the most severe leaves being thread-like and abaxialized. *Rld1-O/+* plants (D) have a normal stature but their leaves are curled upwards because of a partial inversion of adaxial/abaxial polarity. *lbl1-ref Rld1-O* double mutant leaves (E,F) appear normal or display very mild *lbl1-* or *Rld1-O*-like phenotypes, but their subtending internodes elongate normally. The *lbl1-rgd1 Rld1-O* phenotypes (G) are also less severe than either single mutant and frequently resemble the weaker phenotypes of *lbl1-ref*. Detached leaves (H) illustrate the range of leaf phenotypes observed. (H1) wild type; (H2) *lbl1-ref*; (H3) *Rld1-O*; (H4) *lbl1-ref Rld1-O*, note the reduced rolling of the proximal leaf blade near the ligule/auricle when compared with the *Rld1-O* leaf; (H5) *lbl1-rgd1 Rld1-O*, note the morphological similarity to the *lbl1-ref* leaf. Scale bar: 7.5 inches (190.5 mm).

Subsequent PCR reactions were carried out using standard protocols and the following gene specific primers:

ubiquitin, CTGAAAGACAGAACATAATGAGCACAG and TAAGCTGCCGATGTGCCTGCGTCG;

zyb9, CTACAACCGCTTCATCAAGG and AGGTACCATCAGTAGCAAGC;

zyb14, CGACCTCACCGCACGGTCT and GAGCTCCCTCCTGAGTTTGC;

rld1, GAGAGCTAAGAGCAACAAGG and GTTTCTTCAACTAGTGCATGC.

In-situ hybridization and histology

Shoot apices of two-week-old mutant and wild-type sibling seedlings were fixed and embedded as previously described (Jackson, 1991). Tissue sections were pre-treated and hybridized as described by Jackson et al. (Jackson et al., 1994). Digoxigenin-labeled probes comprising the 5′ region including the Zn-finger domain of *zyb9* and *zyb14*, or nucleotides 619-1674 of the *rld1* coding sequence (AY501430), were prepared by in vitro transcription (Stratagene), according to the manufacturer's protocol. *zyb-* and *rld1*-specific probes were used at concentrations of 15 ng/ul/kb and 0.5 ng/ul/kb probe complexity, respectively. Tissue samples for plastic thin sections were fixed overnight at 4°C in a 0.1 M phosphate buffer (pH 7.0) containing 4% glutaraldehyde, dehydrated through an ethanol series, and embedded in JB-4 (Polysciences) according to the manufacturer's protocol. Sections (1 μm) were stained with Toluidine Blue and analyzed under bright field conditions.

Results

Rld1-O suppresses the abaxialization of *lbl1* leaves

lbl1 and *rld1* are both required for the specification of adaxial cell fate. To determine whether these genes act in the same genetic pathway, double mutants between *lbl1* and *Rld1-O* were analyzed. Plants homozygous for the recessive *lbl1-ref* allele display a variety of leaf phenotypes (Timmermans et al., 1998). Mildly phenotypic leaves, resulting from a partial loss of adaxial cell fate, develop patches of abaxial tissue on the adaxial leaf surface. This can result in bifurcation of the leaf or in the formation of fully differentiated lamina at the ectopic abaxial/adaxial boundaries (Fig. 1B, Fig. 1H, part 2). More severely affected *lbl1-ref* leaves, resulting from a complete loss of adaxial identity, become radially symmetric and abaxialized. Such thread-like leaves incorporate fewer founder cells, and consequently have defects in the development and elongation of the subtending internode (Timmermans et al., 1998). As a result, *lbl1-ref* plants have a reduced stature when compared with wild type (Fig. 1A,B; Table 1). The *ragged seedling1* allele of *lbl1* (*lbl1-rgd1*) causes a more severe phenotype. Most *lbl1-rgd1* mutant leaves are narrow or fully abaxialized, and their associated internodes fail to elongate (Fig. 1C). By contrast, SEM and histological analysis showed that *Rld1-O* does not affect founder cell recruitment, even though the loss of miRNA166 regulation in *Rld1-O* affects *rld1* expression in the incipient leaf (Juarez et al., 2004). *Rld1-O* mutants have a normal plant stature and their leaves fully encircle the stem (Fig. 1D, Table 1).

Table 1. The plant height defect of *lbl1-ref* and the ectopic ligule phenotype of *Rld1-O* are both suppressed in the *lbl1-ref Rld1-O* double mutant

Phenotype	Number of plants	Average plant height in inches (mm)	Average number of leaves with ectopic ligule
Wild type	40	85.0±0.8 (2159±20.32)	0
Rld1-O	42	61.4±10.1 (1559.56±256.54)	7.8±1.6**
lbl1-ref	18	38.7±17.8* (982.98±452.12)	0
lbl1-ref Rld1-O	37	60.7±7.2* (1541.78±182.88)	2.4±2.4**

The indicated number of field-grown plants were analyzed at the time of anthesis for both height and the number of leaves with an ectopic abaxial ligule.

*P<0.01; **P<0.005.

The vegetative phenotypes of double mutants homozygous for *lbl1-ref* and heterozygous for *Rld1-O* are variable but much less severe than those observed in *lbl1-ref* single mutant siblings. *lbl1-ref Rld1-O* double mutant leaves frequently appear wild type (Fig. 1E), although partial bifurcations and small ectopic lamina occasionally arise (Fig. 1F). The internode elongation phenotype of *lbl1* is also alleviated in *lbl1-ref Rld1-O* double mutants (Table 1). Consistent with these milder phenotypes, SEM and histological analyses revealed that *lbl1-ref Rld1-O* leaf primordia fully encircle the shoot apex (data not shown). Double mutants between *Rld1-O* and the more severe *lbl1-rgd1* allele also display milder phenotypes than their *lbl1-rgd1* single mutant siblings. Double mutant plants are taller and their leaves resemble the less severe leaves of *lbl1-ref* (Fig. 1G, Fig. 1H, part 5). These results indicate that even though founder cell recruitment is unaffected in *Rld1-O*, altered *rld1* expression in incipient *Rld1-O* primordia can partially suppresses the leaf initiation and adaxial/abaxial polarity defects in *lbl1*.

lbl1 suppresses the adaxialization of *Rld1-O* leaves

rld1 is normally expressed along the adaxial domain and in the midvein region of the P1 leaf. In older leaf primordia, *rld1* expression persists in the vasculature and on the adaxial side near the margins. However, disruption of the miRNA166 complementary site in *Rld1-O* leads to accumulation of *rld1* transcripts on the abaxial side of leaf primordia (Juarez et al., 2004). These changes in *rld1* expression give rise to a variety of adaxial/abaxial polarity defects in both the epidermal and ground tissues, and cause an upward curling of the *Rld1-O* leaf blade (Fig. 1D, Fig. 1H, part 3). The maize leaf comprises a proximal sheath and distal blade region separated by the auricle and ligule (Fig. 2A,B). The ligule is an adaxial epidermal fringe that extends the entire width of the leaf. Approximately half the *Rld1-O* leaves develop patches of ectopic ligule on the abaxial side (Table 1). Such ectopic ligular fringes are usually shorter, arise at a slightly different position along the proximodistal axis, and do not extend the entire width of the leaf (Fig. 2C,D). Sectors of clear tissue often extend proximal and distal from these ectopic ligules. Ground tissue of the wild-type leaf blade consists of evenly spaced longitudinal vascular bundles, which induce the differentiation of concentric rings of photosynthetic bundle sheath and mesophyll cells (Langdale et al., 1988). The clear sectors in *Rld1-O* develop a reduced number of minor lateral veins and transverse veins, and lack the associated photosynthetic cell types (Fig. 2C) (see also Nelson et al., 2002). Such sectors are associated with reduced lateral growth of the leaf blade in addition to the duplication of the ligule, which suggests they coincide with adaxialized regions in the primordium.

Formation of clear sectors is completely suppressed in *lbl1-ref Rld1-O* double mutants, whereas the other *Rld1-O* phenotypes are variably suppressed. Double mutant leaves are often flattened like wild type (Fig. 1E, Fig. 1H, part 5). But in plants where leaves remain curled upwards, this *Rld1-O* phenotype is much less severe, particularly near the tip and base of the leaf blade (Fig. 1F, Fig. 1H, part 4). The number and width of ectopic ligules is also significantly reduced in *lbl1-ref Rld1-O* (Table 1), and no ectopic ligules were observed on fully flattened double mutant leaves, like those shown in

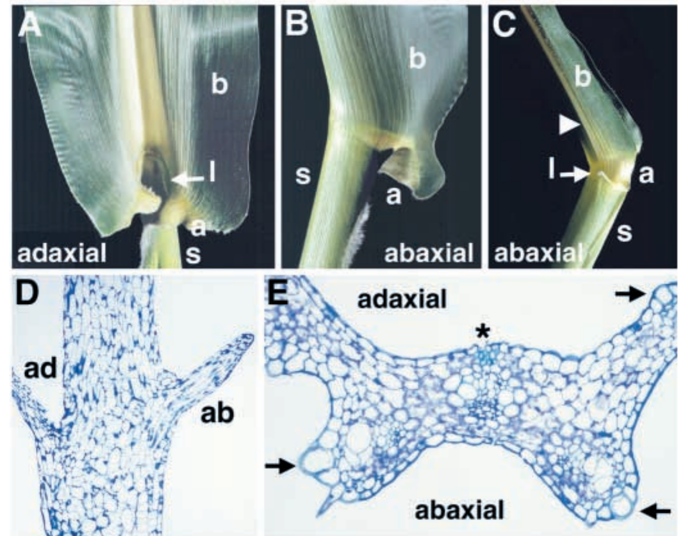

Fig. 2. *Rld1-O* alters adaxial/abaxial polarity in the leaf. The maize leaf (A,B) comprises sheath (s) and blade (b) tissues separated by the auricle (a) and adaxial ligule (l). *Rld1-O* leaves (C) have narrow blades that roll upwards, and frequently develop an abaxial ligule (arrow) and clear sectors with fewer minor veins and no photosynthetic tissue (arrowhead). The ectopic abaxial ligule (D) is narrower, shorter and arises at a slightly different position along the proximodistal axis than the normal adaxial ligule. *Rld1-O* causes a partial inversion in adaxial/abaxial polarity and development of ectopic outgrowths on the abaxial leaf surface (E). Bulliform cells (arrows) are displaced to the abaxial epidermis and schlerenchyma cells (asterisk) develop on the adaxial rather than the abaxial side of intermediate veins. ad, adaxial; ab, abaxial.

Fig. 1E. This suggests that the formation of these *Rld1-O* phenotypes requires normal *lbl1* activity.

Regions of the *Rld1-O* leaf blade surrounding the cleared sectors develop less severe phenotypes. These include the differentiation of schlerenchyma tissue on the adaxial side of intermediate veins rather than the abaxial side, and the formation of small ectopic outgrowths on the abaxial leaf surface (Fig. 2E). The orientation of minor veins is slightly altered near such outgrowths, but development of the ground tissue appears otherwise normal. Therefore, the effect of *Rld1-O* on adaxial/abaxial patterning and its genetic interaction with *lbl1* are most evident in the epidermal layers. The adaxial epidermis of the wild-type leaf blade is characterized by the presence of bulliform cells and macrohairs (Fig. 3A, part 1). All other epidermal cell types, including stomata, microhairs and prickle hairs, are present on both the adaxial and abaxial epidermis (Fig. 3A, part 2). Bulliform cells, like other cells of the epidermis, are arranged in continuous evenly spaced files that run parallel to the underlying vasculature. Macrohairs are regularly distributed within these rows of bulliform cells. Blade tissue adjacent or distal to the clear sectors in *Rld1-O* differentiates macrohairs and bulliform cells on the abaxial rather than the adaxial epidermis (Fig. 3B, parts 1 and 2). This suggests that adaxial/abaxial polarity in the epidermis, like that of the hypodermal schlerenchyma, is partially inverted in *Rld1-O*. Patterning of the adaxial and abaxial epidermal layers is unaffected near the midvein and margins. At the transition from inverted to normal polarity, both the upper and lower leaf

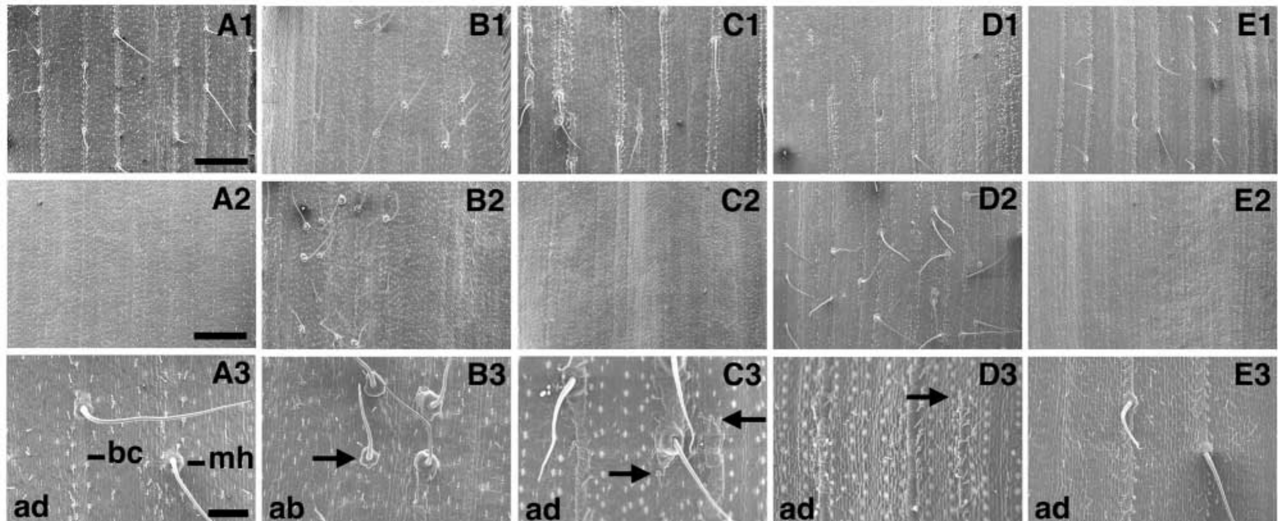

Fig. 3. *lbl1* suppresses the epidermal patterning defects in *Rld1-O*. Scanning electron micrographs of the adaxial (A1-E1) and abaxial (A2-E2) epidermal surfaces of adjacent mature adult leaves samples. (A3-E3) High magnification of selected images to illustrate specific epidermal patterning defects. Compared with wild-type leaves (A), *Rld1-O* leaves (B) display normal polarity near the margins, but, in the center of the lamina, bulliform cells and macrohairs develop on the abaxial surface. Note the presence of isolated macrohairs (arrow in B3) and the overlap between macrohairs on the adaxial and abaxial epidermis. The regular spacing between bulliform cell files and between macrohairs is disrupted in weakly phenotypic *lbl1-ref* leaves (C), and bulliform cell files are frequently disrupted (arrow in C3). *lbl1-ref Rld1-O* leaves with a mild rolled phenotype (D) develop bulliform cells on both the adaxial and abaxial epidermis, and macrohairs on the abaxial epidermis in some regions of the blade. As in *lbl1-ref*, bulliform cell files are discontinuous and irregularly spaced (arrow in D3). Epidermal patterning in *lbl1-ref Rld1-O* leaves with a flattened morphology (E) is indistinguishable from that of wild type. ad, adaxial; ab, abaxial; mh, macrohair; bc, bulliform cell. All samples are oriented with the margin towards the right. Scale bars: in A1-E2, 1 mm; in A3-E3, 0.25 mm.

surfaces differentiate macrohairs in irregularly spaced isolated patches and frequently independently of bulliform cells (Fig. 3B, part 3). The adaxial/abaxial polarity defects in *Rld1-O* thus become progressively less severe towards the margins, midvein and tip of the leaf. Furthermore, duplication of the ligule and macrohairs suggests that misexpression of *rld1* in *Rld1-O* partially adaxializes the primordium, which can lead to abaxialization of the upper leaf surface.

Mild phenotypic *lbl1-ref* leaves develop bulliform cells and macrohairs only in the adaxial epidermis, but their arrangement is disorganized (Fig. 3C, part 1). The bulliform cell files are discontinuous and irregularly spaced, and macrohairs are less evenly spaced within these cell files. The abaxial surface of mild *lbl1-ref* leaves resembles that of wild type (Fig. 3C, part 2), with the exception that margin-associated hairs develop at positions underneath adaxial ectopic laminar outgrowths (data not shown). The adaxial epidermal patterning defects are exacerbated in more severe *lbl1* mutant leaves, whereas the fully abaxialized, threadlike *lbl1* leaves lack both bulliform cells and macrohairs (Timmermans et al., 1998). The polarity of *lbl1-ref Rld1-O* double mutant leaves that display a mild upward curling (Fig. 1F) remains partially inverted; however, the domain of the lamina that is affected is much narrower. Such leaves also display a novel phenotype in that discontinuous patches of bulliform cells without macrohairs develop on the adaxial surface (Fig. 3D, part 1). The pattern of bulliform cells and macrohairs on the abaxial epidermis is also irregular and macrohairs occasionally develop outside the bulliform cell files (Fig. 3D, parts 2 and 3). The effects of *Rld1-O* on the polarity of the leaf, and *lbl1* on the spatial distribution of bulliform cells and macrohairs, are both completely suppressed in *lbl1-ref Rld1-O* double mutant leaves that have a flattened morphology (Fig. 3E, parts 1-3). Thus, such flattened *lbl1-ref Rld1-O* double mutant leaves (Fig. 1E) display none of the *Rld1-O* phenotypes, indicating that *lbl1* can completely suppress the *Rld1-O* phenotype.

The degree of suppression of both the *Rld1-O* and *lbl1* mutant phenotypes in the double mutant is variable and depends largely on the *lbl1* allele and on the expressivity of the *lbl1* mutation during primordium development. To test whether the double mutant phenotype also depends on *Rld1-O* dosage, F2 populations segregating both *lbl1-ref/lbl1-ref Rld1-O/Rld1-O* and *lbl1-ref/lbl1-ref Rld1-O/+* double mutants were analyzed. Double mutants with more severe *Rld1-O* phenotypes did segregate in these families. However, the extreme phenotypic variation observed in these populations made it difficult to conclusively establish whether *lbl1* and *Rld1-O* interact in a dose-dependent manner.

lbl1 acts upstream of *rld1* in the specification of adaxial cell fate

The mutual suppressive interaction between *lbl1* and *Rld1-O* suggests that these genes act in the same genetic pathway. To establish the genetic order in which they act, we analyzed the *rld1* expression pattern in *lbl1-rgd1* apices. *rld1* is normally expressed in the presumptive central zone of the SAM and in a stripe of cells that includes the incipient leaf (Fig. 4A). In the P1 primordium, *rld1* is expressed throughout the adaxial domain but becomes gradually restricted to the adaxial side of the margins during primordium development (Fig. 4B). Loss of adaxial cell fate in *lbl1* is associated with reduced or loss of *rld1* expression on the adaxial side of the leaf (Fig. 4C,D). The

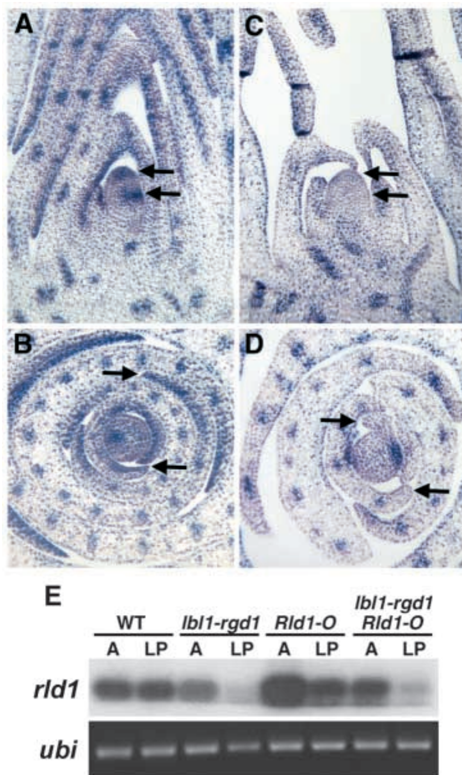

Fig. 4. *lbl1* acts upstream of *rld1*. In situ hybridization shows that *rld1* expression in wild type (A,B) occurs in the SAM and vasculature, and on the adaxial side of leaf primordia. In *lbl1* apices (C,D), *rld1* expression persists in the vasculature but is variably reduced in the SAM and on the adaxial side of developing primordia. Arrows in A and B mark selected regions expressing *rld1*; arrows in C and D mark the absence of *rld1* transcripts in the corresponding positions. (A,C) Longitudinal sections; (B,D) transverse sections. RT-PCR analysis (E) on vegetative apices and young leaf primordia from wild type, *lbl1-rgd1*, *Rld1-O* and the *lbl1 Rld1-O* double mutant indicates that *rld1* transcript levels are reduced in *lbl1-rgd1* but increased in *Rld1-O*. In the double mutant, *rld1* transcripts accumulate to a level intermediate between that of either single mutant. *Ubiquitin* (*ubi*) transcripts were amplified as a control. A, apices comprising the meristem and approximately four young leaf primordia; LP, P5-P8 leaf primordia.

level of *rld1* expression in *lbl1* varies and is negatively correlated with the severity of the *lbl1* mutant. In severe *lbl1-rgd1* mutants, *rld1* expression at the tip of the SAM and at the site of leaf initiation is also lost or reduced, and this coincides with changes in meristem morphology and maintenance (Fig. 4C). These results indicate that *lbl1* acts upstream of *rld1* to specify adaxial cell fate in developing leaf primordia. However, *lbl1* may only indirectly affect *rld1* expression in the SAM. Analysis of the *Antirrhinum phan* and *Arabidopsis* gain-of-function *KANADI* and *YABBY* mutants indicates that abaxialization of the leaf is associated with loss of meristem function (Waites and Hudson, 1995; Siegfried et al., 1999; Eshed et al., 2001).

hd-zipIII genes also play a role in the adaxial/abaxial patterning of vascular bundles (Zhong and Ye, 1999; Ratcliffe et al., 2000; Juarez et al., 2004). *rld1* is expressed in immature vascular strands and becomes localized to the adaxial pro-xylem cells when distinct phloem and xylem poles become apparent (Fig. 4A,B). Interestingly, expression of *rld1* in vascular bundles of the stem and leaf is maintained in *lbl1* (Fig. 4C,D). Even in radially symmetric abaxialized leaves of severe *lbl1* mutants, *rld1* expression persists in the pro-xylem cells (data not shown). This suggests that *lbl1* is specifically required for adaxial/abaxial axis specification during lateral organ development, and that *rld1* acts downstream of *lbl1* in this process, but independently of *lbl1* during vascular patterning.

The reduced expression of *rld1* in *lbl1* was verified by RT-PCR analysis. In wild type, *rld1* transcripts can be detected in the apex, including the SAM and approximately four young leaf primordia, as well as in older leaf primordia (Fig. 4E). *rld1* transcript levels are only moderately reduced in *lbl1* apices, consistent with the residual expression of *rld1* in the vasculature of the stem and young leaf primordia. In older *lbl1* primordia, the levels of *rld1* transcripts are strongly reduced. By contrast, *rld1* expression is increased in both the apex and older leaf primordia of *Rld1-O*. In the *lbl1 Rld1-O* double mutant, *rld1* transcripts accumulate to a level intermediate between that of either single mutant, suggesting that the mutual suppressive interaction between *lbl1* and *Rld1-O* may in part result from their opposing effect on *rld1* expression.

yabby genes are expressed in the adaxial domain of the maize leaf

lbl1 and miRNA166 thus lead to the adaxial specific expression of *rld1* in the leaf. In *Arabidopsis*, downregulation of *HD-ZIPIII* genes allows expression of the *KANADI* and *YABBY* genes, which specify abaxial identity (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001). To further characterize how adaxial/abaxial polarity is established during maize leaf development, homologs of the *Arabidopsis YABBY* genes were isolated and their expression patterns analyzed. Partial genomic fragments from two *yabby* homologs were amplified using degenerate primers designed to conserved motifs in the Zn-finger and YABBY domains of the vegetatively expressed *YABBY* genes *FIL*, *YAB2* and *YAB3* (Sawa et al., 1999; Siegfried et al., 1999). These fragments subsequently allowed the isolation of four full-length *Zea mays yabby* (*zyb*) cDNA clones from a vegetative shoot apex cDNA library. *zyb9* and *zyb10* share 85% nucleotide sequence identity and map to homeologous regions on chromosome arms 5S and 1L, respectively. Two additional *zyb* genes, *zyb14* and *zyb15*, share 71% nucleotide identity, and map to chromosome arms 10L and 5L. None of these genes corresponds to *lbl1*, which maps to chromosome arm 6S.

Phylogenetic analysis of the *Arabidopsis* YABBY proteins indicates that *FIL* and *YAB3* represent a relatively recent gene duplication in the family. The *YAB2* and *YAB5* genes are, in turn, more closely related to *FIL* and *YAB3* than to *CRABSCLAW* (*CRC*) or *INNER NO OUTER* (*INO*) (Siegfried et al., 1999) (Fig. 5). In addition to the Zn-finger and YABBY domains, FIL and YAB3 display sequence similarity in the C-terminal region. ZYB9/10 and ZYB14/15 are also highly conserved in the Zn-finger and YABBY domains, and in the region downstream of the YABBY domain, but the regions between the Zn-finger and the YABBY domains are more diverged. Sequence comparisons between the maize and *Arabidopsis* YABBY proteins suggest that all four maize genes are most closely related to *FIL* and *YAB3*, although the precise

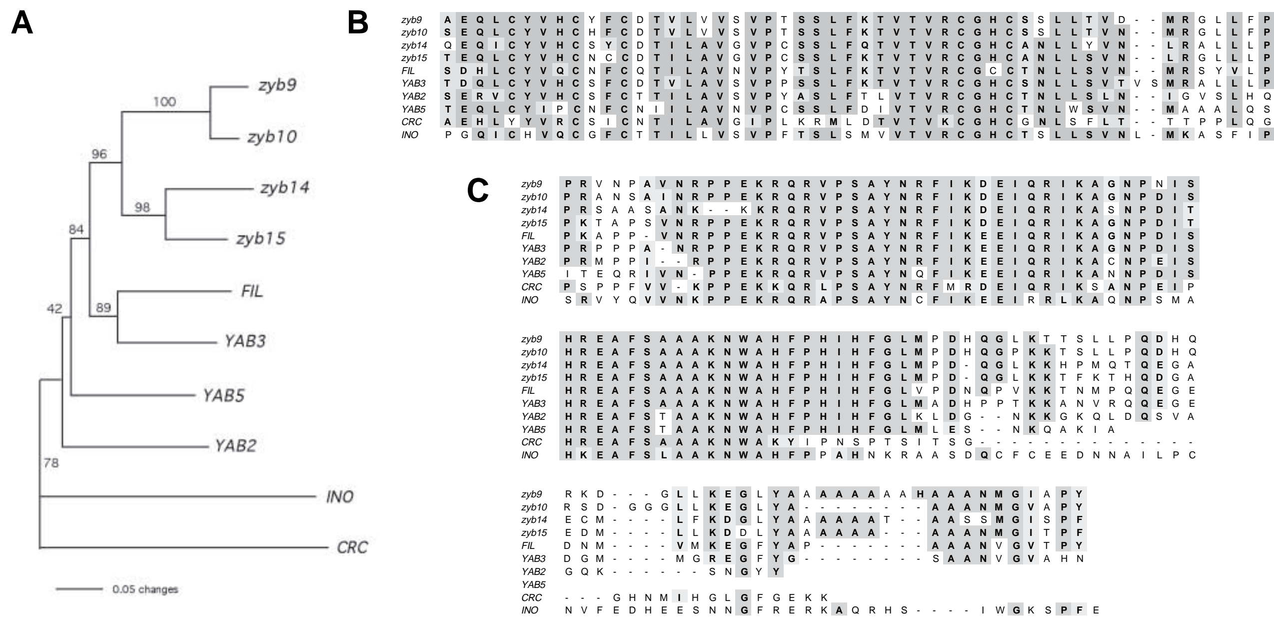

Fig. 5. The maize *yabby* genes, *zyb9*, *10*, *14* and *15*, are homologs of *FIL* and *YAB3*. (A) Phylogenetic analysis of the *Arabidopsis* and maize YABBY proteins demonstrates that the maize *yabby* genes form a separate clade that is most closely related to *FIL* and *YAB3*. Bootstrap values based on 1000 repetitions are indicated. Amino acid sequence alignments of the Zn-finger domains (B), and of the YABBY and C-terminal domains (C), used in the phylogenetic analysis of the maize and *Arabidopsis* YABBY proteins highlight the high level of sequence conservation in these domains of all YABBY proteins. Identical amino acids are shaded dark gray; similar amino acids are shaded light gray. GenBank Accession numbers are: *zyb9*, AY313903; *zyb10*, AY313904; *zyb14*, AY313901; *zyb15*, AY313902.

orthologous relationships between these family members are still unclear (Fig. 5). The divergence between ZYB9/10 and ZYB14/15 is comparable to the divergence between FIL and YAB3, but the maize proteins form a separate clade from FIL and YAB3.

We examined the expression patterns of *zyb9* and *zyb14*, which are the most distantly related family members identified, in wild-type vegetative apices. The vegetatively expressed *YABBY* genes from *Arabidopsis* all have comparable expression patterns. They are expressed throughout the incipient primordium, but, as the leaf emerges, expression becomes restricted to the abaxial side (Sawa et al., 1999; Siegfried et al., 1999). Both *zyb9* and *zyb14* (hence referred to as *yabby* genes) are also expressed in the incipient primordium (Fig. 6). However, their domain of expression appeared smaller than the normal incipient primordium defined by the loss of *knotted1* (*kn1*) expression (Fig. 6A). In order to determine the precise *zyb9* and *zyb14* expression domains within the incipient primordium, adjacent longitudinal sections were hybridized with *kn1* and either *yabby* gene. *kn1* expression is downregulated in at least six tiers of cells (Fig. 6C,E). Surprisingly, expression of both *yabby* genes is limited to the adaxial three tiers of cells (Fig. 6B,D). This suggests that *zyb9* and *zyb14* may function in adaxial/abaxial patterning, but, if so, their function appears to have diverged between maize and *Arabidopsis*, despite the high sequence conservation.

Both *yabby* genes remain preferentially expressed on the adaxial side of P1 leaf primordia, although expression of *zyb9* comprises a slightly broader domain than that of *zyb14* (Fig. 7B,D). In older leaf primordia, both *yabby* genes are expressed in a more restrictive pattern (Fig. 7A-D). Expression near the margins persists throughout the adaxial domain, but, in the remainder of the leaf, expression becomes limited to just the central layer of the ground tissue. This expression pattern could suggest that *zyb9* and *zyb14* become localized to the boundary between the adaxial and abaxial domains of developing leaves. However, because cells near the margins and in the internal layer of the ground tissue, which gives rise to new vascular bundles, differentiate relatively late during primordium development, this expression pattern could also suggest that *yabby* expression is limited to less determined cells of the primordium. In addition, *zyb9* expression also persists in the

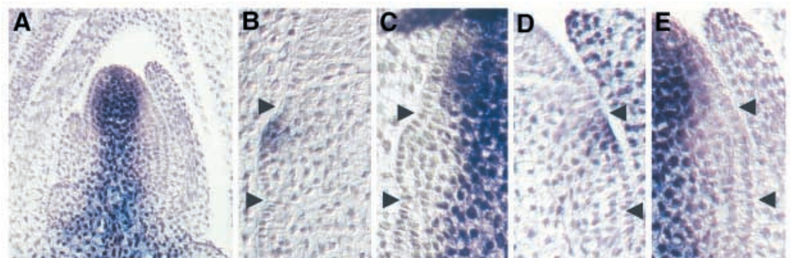

Fig. 6. Maize *yabby* genes are expressed on the adaxial side of incipient leaf primordia. (A) In situ hybridization with a *knotted1* (*kn1*) specific probe shows expression in the meristematic cells of the SAM. (B-E) Hybridization of adjacent longitudinal sections with *kn1* (B) and *zyb14* (C), or *kn1* (D) and *zyb9* (E), shows that incipient leaf primordia defined by lack of *kn1* expression comprise around six tiers of cells (indicated by arrowheads), and that *zyb9* and *zyb14* expression is limited to the adaxial side.

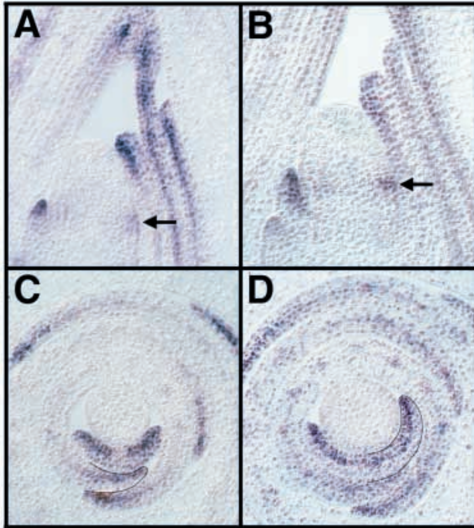

Fig. 7. Maize *yabby* genes are expressed on the adaxial side of leaf primordia. (A-D) In situ hybridization of *zyb14* (A,C) and *zyb9* (B,D) shows that both genes are expressed on the adaxial side of incipient (arrows in A,B) and young leaf primordia. During primordium development expression of *zyb9* and *zyb14* becomes restricted to the margins and to the central layer of the ground tissue. Expression of *zyb9* also persists in the vasculature (D). The black lines outline the margins of some primordia. (A,B) Longitudinal sections; (C,D) transverse sections.

vasculature, indicating that *zyb9* and *zyb14* have some distinct functions during leaf development.

yabby genes act downstream of *lbl1* and *rld1*

In *Arabidopsis*, *YABBY* expression is altered in mutants affecting adaxial/abaxial organ polarity and remains correlated with abaxial cell fate (Siegfried et al., 1999; Eshed et al., 2001). To determine whether *zyb9* and *zyb14* act in the same genetic pathway as *lbl1* and *rld1*, we examined their expression patterns in *lbl1-rgd1* and *Rld1-O*. *lbl1-rgd1* mutants often form narrow, asymmetric leaf primordia that fail to recruit founder cells from the entire circumference of the SAM. In normal margins of *lbl1-rgd1* primordia, expression of both *yabby* genes remains localized to the adaxial side, but the expression level is frequently reduced (Fig. 8A-C, data not shown). Also expression in the internal layer of the ground tissue is reduced in *lbl1-rgd1*, and no *zyb9* or *zyb14* expression was observed in cells at the mutant margins. The expression patterns of both *yabby* genes appeared unaffected in *Rld1-O* introgressed into the B73 inbred line (data not shown). Expression of *zyb9* and *zyb14* was therefore also analyzed in *Rld1-O* introgressed into A158. *Rld1-O* defects are enhanced in this inbred background, relative to B73, and include the formation of multiple ectopic blade outgrowths on immature leaf primordia. Nonetheless, the *zyb9* and *zyb14* expression patterns in *Rld1-O* resembled that in wild type (Fig. 8A,E). Both genes are initially expressed throughout the adaxial domain and their expression persists in the central ground tissue layer and on the adaxial side near the margins in older *Rld1-O* primordia. The minor affect of *Rld1-O* on adaxial/abaxial polarity during early leaf development and near the margins may be consistent with the wild-type *yabby* expression patterns. However, the lack of *zyb9* and *zyb14* expression on the abaxial side in older partially adaxialized *Rld1-O* primordia suggest that these *yabby* genes may not be required for adaxial cell fate, even though loss of adaxial identity in *lbl1* is correlated with reduced expression.

Weak *lbl1* leaf primordia develop ectopic blade outgrowths surrounding abaxialized sectors on the adaxial leaf surface (Timmermans et al., 1998). Expression of both *yabby* genes is

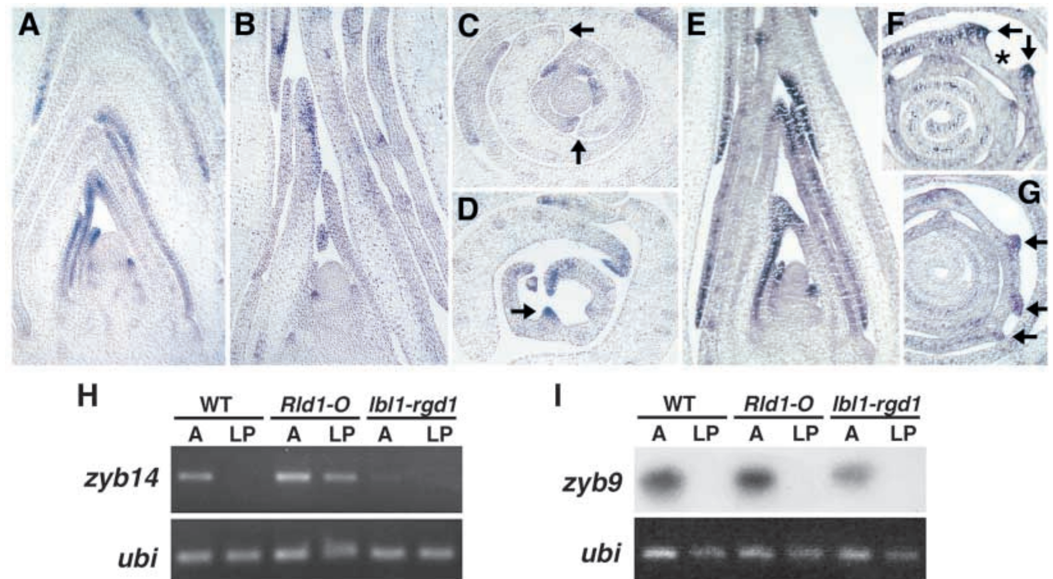

Fig. 8. The *yabby* genes act downstream of *lbl1* and *rld1*. Relative to wild type (A), *zyb14* expression is reduced in *lbl1-rgd1* (B,C). In the narrow *lbl1* leaf primordia, *zyb14* is absent from mutant margins (arrows in C), but remains localized to the adaxial side of normal margins. Expression of *zyb14* is induced uniformly in ectopic lamina on the adaxial surface of *lbl1* leaf primordia (arrow in D). *zyb14* is expressed more abundantly in *Rld1-O* (E), but the pattern of expression in young *Rld1-O* primordia resembles that observed in wild type. Ectopic outgrowths arise on the abaxial surface of *Rld1-O* leaf primordia at the boundary of sectors expressing *zyb14* (F) or *zyb9* (G), and sectors that lack *yabby* expression (e.g. asterisk in F). These abaxial ectopic outgrowths also induce both *zyb14* and *zyb9* expression (arrows in F and G, respectively). RT-PCR analysis on vegetative apices and young leaf primordia from wild type, *lbl1-rgd1*, and *Rld1-O* in the A158 inbred indicates that the relative expression levels of *zyb14* (H) and *zyb9* (I) are reduced in *lbl1-rgd1* apices, and slightly increased in *Rld1-O*. *ubi* transcripts were amplified as a control. A, apices comprising the meristem and approximately four young leaf primordia; LP, P5-P8 leaf primordia.

induced in these ectopic outgrowths, although expression appears uniform, rather than limited to the adaxial side (Fig. 8D). In *Rld1-O*, expression of *zyb9* and *zyb14* persists longer in the internal layer of the ground tissue but expression is not uniform (Fig. 8E-G). Interestingly, ectopic outgrowths develop on the abaxial surface of *Rld1-O* leaf primordia at the apparent boundary between *yabby*-expressing and non-expressing sectors (Fig. 8F,G). Like the adaxial ectopic outgrowths observed in *lbl1*, these abaxial ectopic outgrowths express both *yabby* genes. These results together with the marginal expression during normal primordium development suggest that *zyb9* and *zyb14* expression may be associated with blade outgrowth.

The in situ hybridization signals for both *yabby* genes were more intense in incipient and young *Rld1-O* primordia, suggesting their expression levels may be increased (Fig. 8E). By contrast, the in situ hybridization signals were consistently less intense in *lbl1-rgd1* (Fig. 8B,C). To confirm that these differences in signal intensity reflect altered levels of *zyb9* and *zyb14* expression, their transcript levels in wild type, *lbl1-rgd1* and *Rld1-O* in the A158 inbred background were compared by RT-PCR. Consistent with the in situ hybridization data, *zyb14* is expressed in wild-type apices comprising the SAM and four to five young leaf primordia (Fig. 8H). No transcripts were detected in older leaf primordia. The level of *zyb14* transcripts is strongly reduced in *lbl1-rgd1* apices. By contrast, more *zyb14* transcripts accumulate in *Rld1-O* apices and expression of *zyb14* persists in older leaf primordia. Expression of *zyb9* is also limited to the apex in wild type, *lbl1-rgd1* and *Rld1-O* (Fig. 8I). *zyb9* transcripts are less abundant than *zyb14*, and are only moderately reduced in *lbl1-rgd1* and upregulated in *Rld1-O*. These results suggest that *zyb9* and *zyb14* act downstream of *lbl1* and *rld1*. The mutually suppressive interaction between *lbl1* and *Rld1-O* should therefore be evident in the *yabby* transcript levels. Unfortunately, in the B73 inbred background, which was used to generate the *lbl1 Rld1-O* double mutants, both *lbl1* and *Rld1-O* display relatively mild phenotypes. As a result no significant differences in *yabby* transcript levels were observed in the single and double mutants by RT-PCR (data not shown). Nonetheless, the in situ hybridization and RT-PCR results in A158 suggest that *zyb9* and *zyb14* act downstream of *lbl1* and *rld1*, and may direct mediolateral outgrowth.

Discussion

Rld1 causes a variety of adaxial/abaxial polarity defects

Disruption of the miRNA166 complementary site in *Rld1-O* leads to misexpression of *Rld1* transcripts on the abaxial side of developing leaf primordia (Juarez et al., 2004). This causes a variety of adaxial/abaxial polarity defects in both the epidermal and ground tissues, which become progressively less severe towards the midvein, margins and tip of the leaf. The complex pattern of phenotypes observed in heterozygous *Rld1-O* mutants may, in part, arise from the temporal variation in *Rld1* mutant transcript levels combined with the spatial and temporal variation in the expression of other adaxial determinants, including *phb* and normal *rld1*. Founder cell recruitment, early vascular development and formation of the midrib region, which differentiates in response to signals from the midvein, are unaffected in *Rld1-O*. This indicates that *Rld1-O* leaf primordia initially establish normal polarity despite misexpression of *Rld1* on the abaxial side of incipient and P1 leaf primordia. Adaxial/abaxial polarity may result from remaining differential *hd-zipIII* expression due to accumulation of normal *rld1*, *phb* and potential other *hd-zipIII* transcripts on the adaxial side early in *Rld1-O* leaf development. The progressive expansion of the miRNA166 expression domain gradually restricts normal adaxial *hd-zipIII* expression to the leaf margins, which may consequently maintain normal adaxial/abaxial polarity (Juarez et al., 2004). By contrast, uniform *Rld1* expression persists in the central region of P2-P3 *Rld1-O* primordia. Formation of the clear sectors may be induced by this uniform strong expression of *Rld1*, as it is associated with duplication of the ligule and loss of minor lateral veins, which normally arise during those stages of primordium development (Sharman, 1942; Nelson and Dengler, 1997; Sylvester et al., 1990). Normal development of vascular and photosynthetic cell types outside the clear sectors suggests that the observed gradual reduction in *Rld1* mutant transcripts in older *Rld1-O* primordia is not sufficient to affect polarity in the ground tissue. However, the differentiation of epidermal cell types and schlerenchyma tissue remains affected, suggesting that these tissues require less *Rld1* activity than the internal layers of the leaf to become adaxialized.

Consistent with the possibility that the varied *Rld1-O* phenotypes require different levels of *Rld1* expression, the frequency and size of clear sectors is enhanced in homozygous *Rld1-O* mutants. Moreover, formation of clear sectors is completely suppressed in *lbl1 Rld1-O* double mutants, whereas *lbl1* only partially suppresses the schlerenchyma and epidermal phenotypes. *lbl1* acts upstream of *rld1* in the pathway leading to adaxial identity and is required for the accumulation of *rld1* transcripts in developing leaf primordia. Therefore, *lbl1* is likely to suppress the *Rld1-O* phenotypes by reducing *Rld1* mutant transcript levels in the developing leaf. The severity of *Rld1-O* phenotypes is also suppressed in hyperploid plants that carry an additional normal copy of *rld1* (Nelson et al., 2002), supporting the possibility that phenotypic severity depends on the relative levels of *Rld1* and other adaxial determinants. Mutations that disrupt the miRNA165/166 complementary site in the *Arabidopsis HD-ZIPIII* genes also cause a range of phenotypes. Such dominant alleles of *PHB* and *PHV* cause formation of radially symmetric adaxialized leaves, whereas similar mutations in *REV* mainly affect vascular patterning (McConnell et al., 2001; Emery et al., 2003). Whether these phenotypic differences reflect differences in the relative expression levels of these *HD-ZIPIII* genes during primordium and vascular development remains to be determined.

In addition to the expected adaxialization of the lower leaf surface, the upper blade surface of *Rld1-O* leaves becomes partially abaxialized such that adaxial/abaxial polarity is inverted. None of the gain-of-function alleles of *PHB*, *PHV* or *REV* cause an inversion in polarity, but weak recessive alleles of *ARGONAUTE*, which is required for the miRNA-mediated cleavage of *HD-ZIPIII* transcripts, can invert leaf polarity (Kidner and Martienssen, 2004). Variation in the relative levels of *rld1* and other adaxial determinants during leaf development could also underlie this aspect of the *Rld1-O* phenotype. In *Drosophila*, variation in the relative levels of the ventral determinants, Dorsal and Twist, results in inverted dorsoventral polarity in the embryo (Stathopoulos and Levine, 2002). High

nuclear concentrations of both Dorsal and Twist, induces ventral mesoderm, whereas high levels of Dorsal together with low levels of Twist leads to formation of more dorsal cell types. However, Dorsal also induces ventral identity in the absence of Twist. By analogy, balanced levels of *rld1* and other adaxial determinants may induce adaxial identity. However, the gradual downregulation of such adaxial determinants during primordium development progressively changes their ratio relative to *Rld1*. This may temporarily cause induction of abaxial cell fate but, upon further reduction of adaxial determinants, again lead to specification of adaxial identity. Alternatively, as signaling between the adaxial and abaxial domains is important to coordinate outgrowth and patterning of the leaf, switching of cell identity in the adaxial layer may be a consequence of the adaxialization of the lower leaf surface (see also Nelson et al., 2002).

lbl1 specifies adaxial fate during lateral organ development via *rld1*

lbl1 is required for the specification of adaxial cell fate in lateral organs (Timmermans et al., 1998). Loss of *lbl1* activity affects lateral founder cell recruitment in addition to adaxial/abaxial patterning, and both these defects are suppressed in the double mutant with *Rld1-O*. Expression of *zyb9* and *zyb14* is increased on the adaxial side of incipient and young *Rld1-O* primordia, which could counteract the reduced expression of these *yabby* genes in *lbl1*. But, why are the *yabby* expression levels increased in *Rld1-O*? miRNA166 only accumulates on the abaxial side in the incipient and P1 leaf (Juarez et al., 2004). Therefore, increases in adaxial *yabby* expression levels must arise independently of the loss of miRNA166 action in *Rld1-O*. In situ hybridization intensities suggest that *rld1* expression is similarly upregulated on the adaxial side in *Rld1-O* prior to the accumulation of miRNA166. Similarly, disruption of the miRNA165/166 complementary site in *PHB* causes overexpression of *PHB* transcripts on the adaxial side in addition to ectopic expression of mutant transcripts on the abaxial side (McConnell et al., 2001). This increase in adaxial expression also precedes the accumulation of miRNA165 in that domain (Kidner and Martienssen, 2004). The adaxial domain of the leaf promotes meristem function (McConnell and Barton, 1998; Kidner et al., 2002). Conversely, specification of adaxial cell fate requires a signal from the meristem (Sussex, 1951; Sussex, 1955). Owing to such reciprocal communication between the SAM and the leaf, the production, perception or activity of the meristem-borne signal may be altered in the adaxialized *Rld1-O* and *phb-1d* mutants. HD-ZIPIII proteins contain a highly conserved START lipid-sterol binding domain, and potentially become activated in response to the meristem-derived signal. As a result, genes acting downstream of the *hd-zipIII* genes, such as *zyb9* and *zyb14*, may become upregulated. Moreover, if *hd-zipIII* genes are positively autoregulated, adaxial *hd-zipIII* expression can be increased independent of the loss of miRNA166 directed transcript cleavage.

lbl1 specifies adaxial identity by regulating the accumulation of *rld1* transcripts on the adaxial side of developing leaf primordia. *rld1* and *phb* have similar expression patterns and probably act redundantly, as the *HD-ZIPIII* genes do in *Arabidopsis* (Emery et al., 2003). Loss of adaxial identity in *lbl1* mutants thus suggests that *lbl1* not only acts upstream of *rld1* but possibly upstream of other *hd-zipIII* genes as well. Meristematic expression of *rld1* is also reduced in *lbl1*. However, this could result from reduced adaxial identity in adjacent leaf primordia rather than from a direct effect of *lbl1* on *hd-zipIII* expression in the SAM. The level and pattern of *rld1* expression in the vasculature of *lbl1* is not affected, which makes it unlikely that *lbl1* controls *hd-zipIII* expression by modulating the miRNA166 expression domain. Transcription of the *hd-zipIII* genes may depend on *lbl1* function directly. Alternatively, if *hd-zipIII* expression is autoregulated in a ligand-dependent manner, *lbl1* may affect the accumulation of *hd-zipIII* transcripts indirectly by regulating the production or perception of this ligand. The radially symmetric abaxialized leaves that arise following surgical separation from the SAM are shorter than normal and develop siphonostelic (with phloem and xylem cells surrounding a central pith) or protostelic (with phloem surrounding xylem) vascular bundles (Sussex, 1951; Sussex, 1955). Depending on expressivity, *lbl1* leaves display comparable growth and patterning defects (Timmermans et al., 1998) (M.T.J. and M.C.P.T., unpublished).

Specification of adaxial/abaxial polarity during vascular and lateral organ development involves a partially conserved mechanism. *rld1* and *phb* expression on the adaxial side of lateral organs, and in the adaxial pro-xylem cells, are both defined by the pattern of miRNA166 accumulation (Juarez et al., 2004). In *Arabidopsis*, *KANADI* genes are expressed on the abaxial side of developing organs, and vascular expression is limited to the abaxial and peripheral phloem cells (Kerstetter et al., 2001; Emery et al., 2003). Mutational analysis has further shown that the *KANADI* and *HD-ZIPIII* genes act antagonistically during both vascular and lateral organ development (Emery et al., 2003). The miRNA-directed cleavage of *HD-ZIPIII* transcripts is conserved throughout all lineages of land plants and precedes the origin of angiosperm leaves (Floyd and Bowman, 2004). Therefore, the *MIR166*, *HD-ZIPIII* and, possibly, the *KANADI* genes may have had an initial role in the specification of adaxial/abaxial polarity in the vascular tissue of non-leafy plants, only later acquiring an additional function in the patterning of lateral organs (Eshed et al., 2001; Kidner et al., 2002; Emery et al., 2003). Because *lbl1* affects *hd-zipIII* expression only on the adaxial side of lateral organs and not in the vasculature, its role in adaxial/abaxial patterning could coincide with and possibly contribute to the derivation of leaves from branching shoots (Gifford and Foster, 1989).

Maize *yabby* genes may direct lateral outgrowth rather than specify adaxial cell fate

Loss- and gain-of-function mutations reveal a role for *YABBY* genes in the specification of abaxial cell fate in *Arabidopsis* (Sawa et al., 1999; Siegfried et al., 1999; Kumaran et al., 2002). Consistent with this role, *YABBY* gene expression is correlated with the abaxial domain in wild-type and mutant leaf primordia (Siegfried et al., 1999; Eshed et al., 2001). The tomato *FIL/YAB3* homolog, *LeYAB B*, is similarly expressed on the abaxial side of leaf primordia, and may function in the specification of abaxial cell identity in this compound-leaved species (Kim et al., 2003). *zyb9* and *zyb14* are expressed in a polar pattern, but, unlike *Arabidopsis* and tomato, these maize *yabby* genes are expressed on the adaxial side of incipient and young leaf primordia. Because *rld1* and *phb* are expressed in

a pattern analogous to the *Arabidopsis HD-ZIPIII* genes on the adaxial side of developing leaves, the regulation and/or function of the *yabby* genes must have diverged between *Arabidopsis* and maize. The *Arabidopsis HD-ZIPIII* genes suppress *YABBY* expression on the adaxial side of P2 and older leaf primordia (Eshed et al., 2001). By contrast, expression of *zyb9* and *zyb14* mirrors that of the *hd-zipIII* genes, and their increased expression in *Rld1-O* indicates that both *yabby* genes are positively regulated by *rld1*. Maize *yabby* expression persists outside the *hd-zipIII* expression domain at the presumptive adaxial/abaxial boundary, and misexpression of *Rld1* is not sufficient to induce *zyb9* and *zyb14* expression on the abaxial side during *Rld1-O* primordium development. These observations suggest that other factors in addition to the *hd-zipIII* genes control *yabby* gene expression.

YABBY function may also have diverged between *Arabidopsis* and maize despite the high amino acid sequence conservation. Specification of adaxial/abaxial polarity leads to mediolateral outgrowth and patterning of the leaf and both of these processes are affected in *fil yab3* (Siegfried et al., 1999; Kumaran et al., 2002). The role of the maize *yabby* genes in leaf development is less clear. Transposon insertion alleles of *zyb9* and *zyb14* display no phenotypes, probably because of functional redundancy (M.T.J. and M.C.P.T., unpublished). *yabby* genes may specify adaxial identity, as reduced adaxial cell fate in *lbl1* is correlated with decreased *zyb9* and *zyb14* expression. However, adaxialization of *Rld1-O* leaf primordia is not correlated with *yabby* expression on the abaxial side. Also, their apparent uniform expression in the *lbl1* ectopic outgrowths is inconsistent with a role for *zyb9* and *zyb14* in adaxial cell fate determination. The expression patterns of these maize *yabby* genes suggest that they may function during mediolateral outgrowth. The *lbl1* defect in founder cell recruitment is correlated with reduced *zyb9* and *zyb14* expression, and suppression of this defect in *lbl1 Rld1-O* is associated with increased *yabby* expression in the incipient primordium. Also, ectopic outgrowths in *lbl1* and *Rld1-O* express both *yabby* genes, irrespective of whether such outgrowths arise on the adaxial or abaxial side of the leaf.

Ectopic lamina on weakly phenotypic *lbl1* leaves arise at the boundary of abaxialized sectors on the adaxial leaf surface (Timmermans et al., 1998). In *Rld1-O*, no ectopic outgrowths develop at the boundaries of regions with inverted polarity, suggesting that juxtaposition of adaxial and abaxial cells in just the epidermis and subepidermal schlerenchyma is insufficient to induce lateral outgrowth. Interestingly, ectopic outgrowths in *Rld1-O* arise on the abaxial side at positions where blade tissue expressing *zyb9* and *zyb14* in the central layer of the ground tissue is juxtaposed next to blade tissue that no longer expresses these *yabby* genes. The polar expression of the *yabby* genes in the incipient primordium and at the margins of older leaf primordia may similarly be required for founder cell recruitment and continued mediolateral blade outgrowth. Lateral outgrowth during *Arabidopsis* primordium development is also correlated with polar *YABBY* gene expression. *FIL* and *YAB3* are uniformly expressed in the incipient primordium but become restricted to the abaxial side at the time blade outgrowth occurs (Sawa et al., 1999; Siegfried et al., 1999). However, *lbl1* ectopic lamina initially show uniform expression of *zyb9* and *zyb14*. Perhaps, juxtaposition of *yabby* expressing and non-expressing cells is not essential for lateral outgrowth in that context, or perhaps outgrowth of ectopic blade tissues is initially restricted to their base.

The maize and *Arabidopsis yabby* genes may thus share a role in mediolateral outgrowth. However, *Arabidopsis YABBY* genes also play a role in abaxial cell fate determination (Sawa et al., 1999; Seigfried et al., 1999; Kumaran et al., 2002). Although the distinct phenotypes of *kan1 kan2* and *fil yab3* mutants and their epistatic interactions suggest that the *KANADI* and *YABBY* genes act in separate pathways with both distinct and overlapping targets (Eshed et al., 2001). Mediolateral growth of the maize leaf is initiated within the context of positional information inherent in the meristem, whereas lateral blade outgrowth in *Arabidopsis* occurs after emergence of the primordium from the SAM. Owing to these distinct growth habits, maize and *Arabidopsis yabby* genes may be under different evolutionary constraints. *YABBY* genes in *Arabidopsis* may have a specific role in the maintenance of the meristematic positional information in the isolated primordium that is not required in maize. In the absence of such a requirement, selection to maintain a specific polar expression pattern could be weakened. Most monocots elaborate dorsoventral blade tissue, like maize does, from the lower leaf zone by lateral founder cell recruitment. However, several monocot species that develop unifacial leaves or that develop blade tissue from the upper leaf zone after primordium emergence, like *Arabidopsis* does, are nested within the monocot clade (Kaplan, 1973; Bharathan, 1996). Comparative analysis of *yabby* expression patterns in such diverse monocots may help to elucidate whether *yabby* genes are indeed under different evolutionary constraints depending on the leaf growth habit.

Adaxial/abaxial axis specification in the maize leaf

lbl1, *rld1*, and the *mir166* and *yabby* genes act in the same genetic pathway leading to adaxial cell fate and mediolateral outgrowth of the leaf (Fig. 9). *rld1* in combination with other regulatory factors leads to adaxial expression of the *yabby* genes *zyb9* and *zyb14*. Polarized expression of these *yabby* genes may mediate lateral founder cell recruitment and thus, directly or indirectly, control the downregulation of *knox* genes. *rld1* also specifies adaxial cell fate but probably independently of the *yabby* genes. Adaxial-specific expression of *rld1* in the developing leaf depends on *lbl1* and miRNA166.

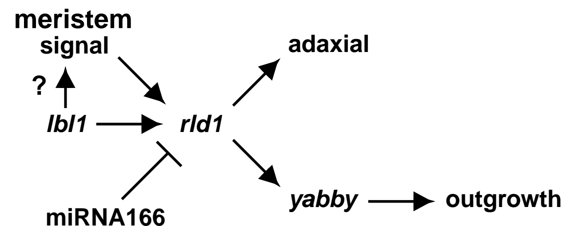

Fig. 9. Genetic pathway leading to adaxial cell fate and mediolateral growth of the maize leaf. *lbl1* and miRNA166 have an opposing affect on the accumulation of *rld1* transcripts, and together lead to the adaxial specific expression of *rld1*. RLD1 specifies adaxial cell fate, possibly upon activation by the proposed meristem-derived signal. RLD1 also induces *yabby* gene expression in the adaxial domain, and the juxtaposition of *yabby*-expressing and non-expressing cells mediates mediolateral outgrowth.

lbl1 positively affects the accumulation of *rld1* transcripts, whereas miRNA166 directs their cleavage. miRNA166 initially accumulates immediately below the incipient leaf but gradually spreads via the abaxial side throughout the developing primordium (Juarez et al., 2004). The specification of adaxial cell fate also requires a signal from the meristem (Sussex, 1951; Sussex, 1955). This signal could act via RLD1 and other HD-ZIPIII family members, as they contain a START lipid-sterol binding domain. If so, RLD1 and other HD-ZIPIII proteins may specify adaxial/abaxial polarity in developing leaves by incorporating positional information established by two opposing signals that originate outside the incipient primordium: the adaxializing signal from the SAM and the miRNA166 signal from a potential signaling center below the incipient leaf. Finally our results present the possibility that *lbl1* specifies adaxial cell fate in developing leaf primordia by altering the production or perception of the proposed meristem-borne signal.

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