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 side. 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.

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 KANADI1 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 adaxial 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-ZIP III 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 a 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 (lbb1) activity. Recessive mutations in lbb1 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 lbb1 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 lbb1 and Rld1-0 mutually suppress each other, and that lbb1 is required for normal rld1 expression in the SAM and on the adaxial side of leaf primordia. lbb1 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 lbb1 mutants, suggesting that adaxial/abaxial polarity in veins may be established independently of lbb1 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 lbb1 and Rld1-O mutants suggest they act downstream of lbb1 and rld1, and may direct lateral organ outgrowth. These observations suggest that lbb1, 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, CA) and like lbb1-ref and lbb1-rgd1 introgressed three to six times into diverse inbred backgrounds. Both the lbb1-ref and lbb1-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 lbb1 and heterozygous for Rld1-O were generated in the B73 inbred line by backcrossing lbb1-ref y1/+; Rld1-0/ + or lbb1-rgd1 y1/+; Rld1-0/ + double heterozygous plants to heterozygous lbb1-ref y1/+ and lbb1-rgd1 y1/+ siblings, respectively. Several hundred white (y1y1/y1) and dark yellow (++/+y1) 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 lbb1 single and lbb1 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 lbb1 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 ethanola 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’ (TGCTAGTSMAMTGCARC-WYTG) and YAB3’ (RTYYTTNGCWGCAGYCRRAAKGC), 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 recombiant 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 μg of DNaseI-treated RNA was primed with oligo(dT) and converted to complementary DNA using M-MULV reverse transcriptase (NEB).
Subsequent PCR reactions were carried out using standard protocols and the following gene specific primers: ** ubiquitous, CTGAAAGACAGACATATAACGACACG and TAA-GCTGCCGATGCTGCTGTCG;** 

zyb9, CTACAACCGTCTCATACAG and AGGTCCCATCAC- TAGCAAGC; 

zyb9, CGACCTACCCACCTACAGTT and GAGGCTCTCTC-GAGATTGC; 

rld1, GAGAGCTAGAGCAACAAGG and GTTTCTTCACTAGTGCATG.

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 zyb14 coding sequence (AYSO1430), 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


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

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 \( \text{lbl1-ref} \) and heterozygous for \( \text{Rld1-O} \) are variable but much less severe than those observed in \( \text{lbl1-ref} \) single mutant siblings. \( \text{lbl1-ref} \) \( \text{Rld1-O} \) 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 \( \text{lbl1} \) is also alleviated in \( \text{lbl1-ref} \) \( \text{Rld1-O} \) double mutants (Table 1). Consistent with these milder phenotypes, SEM and histological analyses revealed that \( \text{lbl1-ref} \) \( \text{Rld1-O} \) leaf primordia fully encircle the shoot apex (data not shown). Double mutants between \( \text{Rld1-O} \) and the more severe \( \text{lbl1-rgd1} \) allele also display milder phenotypes than their \( \text{lbl1-rgd1} \) single mutant siblings. Double mutant plants are taller and their leaves resemble the less severe leaves of \( \text{lbl1-ref} \) (Fig. 1G, Fig. 1H, part 5). These results indicate that even though founder cell recruitment is unaffected in \( \text{Rld1-O} \), altered \( \text{rld1} \) expression in incipient \( \text{Rld1-O} \) primordia can partially suppresses the leaf initiation and adaxial/abaxial polarity defects in \( \text{lbl1} \).

**\( \text{lbl1} \) suppresses the adaxialization of \( \text{Rld1-O} \) leaves**

\( \text{rld1} \) is normally expressed along the adaxial domain and in the midvein region of the P1 leaf. In older leaf primordia, \( \text{rld1} \) expression persists in the vasculature and on the adaxial side near the margins. However, disruption of the miRNA166 complementary site in \( \text{Rld1-O} \) leads to accumulation of \( \text{rld1} \) transcripts on the abaxial side of leaf primordia (Juarez et al., 2004). These changes in \( \text{rld1} \) 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 \( \text{Rld1-O} \) 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 \( \text{Rld1-O} \) 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 is characterized by the differentiation of concentric rings of photosynthetic bundle sheath and mesophyll cells (Langdale et al., 1988). The clear differentiation of concentric rings of photosynthetic bundle sheath and mesophyll cells (Langdale et al., 1988). Ground tissue adjacent or distal to the clear sectors in \( \text{Rld1-O} \) phenotypes are variably suppressed. Double mutant leaves are frequently flattened double mutant leaves, like those shown in Fig. 1E. This suggests that the formation of these \( \text{Rld1-O} \) phenotypes requires normal \( \text{lbl1} \) activity.

Regions of the \( \text{Rld1-O} \) 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. The orientation of minor veins is slightly altered near such outgrowths, but development of the ground tissue appears otherwise normal. Therefore, the effect of \( \text{Rld1-O} \) on adaxial/abaxial patterning and its genetic interaction with \( \text{lbl1} \) 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 \( \text{Rld1-O} \) 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 \( \text{Rld1-O} \). 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. 

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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-rld1* 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
level of \textit{rld1} expression in \textit{lbl1} varies and is negatively correlated with the severity of the \textit{lbl1} mutant. In severe \textit{lbl1-rgd1} mutants, \textit{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 \textit{lbl1} acts upstream of \textit{rld1} to specify adaxial cell fate in developing leaf primordia. However, \textit{lbl1} may only indirectly affect \textit{rld1} expression in the SAM. Analysis of the \textit{Antirrhinum phan} and \textit{Arabidopsis} gain-of-function \textit{KANADI} and \textit{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).

\textit{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). \textit{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 \textit{rld1} in vascular bundles of the stem and leaf is maintained in \textit{lbl1} (Fig. 4C,D). Even in radially symmetric abaxialized leaves of severe \textit{lbl1} mutants, \textit{rld1} expression persists in the pro-xylem cells (data not shown). This suggests that \textit{lbl1} is specifically required for adaxial/abaxial axis specification during lateral organ development, and that \textit{rld1} acts downstream of \textit{lbl1} in this process, but independently of \textit{lbl1} during vascular patterning.

The reduced expression of \textit{rld1} in \textit{lbl1} was verified by RT-PCR analysis. In wild type, \textit{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). \textit{rld1} transcript levels are only moderately reduced in \textit{lbl1} apices, consistent with the residual expression of \textit{rld1} in the vasculature of the stem and young leaf primordia. In older \textit{lbl1} primordia, the levels of \textit{rld1} transcripts are strongly reduced. By contrast, \textit{rld1} expression is increased in both the apex and older leaf primordia of \textit{Rld1-O}. In the \textit{lbl1 Rd1-O} double mutant, \textit{rld1} transcripts accumulate to a level intermediate between that of either single mutant, suggesting that the mutual suppressive interaction between \textit{lbl1} and \textit{Rld1-O} may in part result from their opposing effect on \textit{rld1} expression.

\textbf{yabby genes are expressed in the adaxial domain of the maize leaf}

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

Phylogenetic analysis of the \textit{Arabidopsis} \textit{YABBY} proteins indicates that \textit{FIL} and \textit{YAB3} represent a relatively recent gene duplication in the family. The \textit{YAB2} and \textit{YAB5} genes are, in turn, more closely related to \textit{FIL} and \textit{YAB3} than to \textit{CRABSCLAW (CRC)} or \textit{INNER NO OUTER (INO)} (Siegfried et al., 1999) (Fig. 5). In addition to the \textit{Zn}-finger and \textit{YABBY} domains, \textit{FIL} and \textit{YAB3} display sequence similarity in the C-terminal region. \textit{Zyb9}/10 and \textit{Zyb14}/15 are also highly conserved in the \textit{Zn}-finger and \textit{YABBY} domains, and in the region downstream of the \textit{YABBY} domain, but the regions between the \textit{Zn}-finger and the \textit{YABBY} domains are more diverged. Sequence comparisons between the maize and \textit{Arabidopsis} \textit{YABBY} proteins suggest that all four maize genes are most closely related to \textit{FIL} and \textit{YAB3}, although the precise
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...
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

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 sclerenchyma 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 sclerenchyma 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 Rldl. 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).

**lb1** specifies adaxial fate during lateral organ development via rld1

**lb1** is required for the specification of adaxial cell fate in lateral organs (Timmermans et al., 1998). Loss of **lb1** activity affects lateral founder cell recruitment in addition to adaxial/abaxial patterning, and both these defects are suppressed in the double mutant with **Rldl-O**. Expression of zyb9 and zyb14 is increased on the adaxial side of incipient and young **Rldl-O** primordia, which could counteract the reduced expression of these **yabby** genes in **lb1**. But, why are the **yabby** expression levels increased in **Rldl-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 **Rldl-O**. In situ hybridization intensities suggest that **rld1** expression is similarly upregulated on the adaxial side in **Rldl-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 **yabby** 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 **Rldl-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.

**lb1** 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 **lb1** mutants thus suggests that **lb1** not only acts upstream of **rld1** but possibly upstream of other **hd-zipIII** genes as well. Meristematic expression of **rld1** is also reduced in **lb1**. However, this could result from reduced adaxial identity in adjacent leaf primordia rather than from a direct effect of **lb1** on **hd-zipIII** expression in the SAM. The level and pattern of **rld1** expression in the vasculature of **lb1** is not affected, which makes it unlikely that **lb1** controls **hd-zipIII** expression by modulating the miRNA166 expression domain. Transcription of the **hd-zipIII** genes may depend on **lb1** function directly. Alternatively, if **hd-zipIII** expression is autoregulated in a ligand-dependent manner, **lb1** 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, **lb1** 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 **lb1** 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 FLY/AB3 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-ZIP III 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-ZIP III genes suppress YABBY expression on the adaxial side of P2 and older leaf primordia (Eshed et al., 2001). By contrast, expression of zfb9 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 zfb9 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 zfb9 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 zfb9 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 zfb9 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 zfb9 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 sclerenchyma is insufficient to induce lateral outgrowth. Interestingly, ectopic outgrowths in Rld1-O arise on the abaxial side at positions where blade tissue expressing zfb9 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 zfb9 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; Siegfried 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 do, 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 zfb9 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.
lb1 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 lb1 specifies adaxial cell fate in developing leaf primordia by altering the production or perception of the proposed meristem-born signal.

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