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Expression of a mutant maize gene in the ventral leaf epidermis is sufficient to signal a switch of the leaf's dorsoventral axis

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SUMMARY

Maize leaves are initiated from the shoot apex with an inherent leaf dorsoventral polarity; the leaf surface closest to the meristem is the adaxial (upper, dorsal) surface whereas the opposite leaf surface is the abaxial (lower, ventral) surface. The *Rolled leaf1* (*Rld1*) semi-dominant maize mutations affect dorsoventral patterning by causing adaxialization of abaxial leaf regions. This adaxialization is sometimes associated with abaxialization of the adaxial leaf regions, which constitutes a 'switch'. Dosage analysis indicates *Rld1* mutants are antimorphs. We mapped *Rld1*'s action to a single cell layer using a mosaic analysis and show *Rld1* acts non cell-autonomously along the dorsoventral axis. The presence of *Rld1* mutant product in the abaxial epidermis is necessary and sufficient to induce the Rolled leaf1 phenotype within the lower epidermis as well as in other leaf layers along the dorsoventral axis. These results support a model for the involvement of wild-type RLD1 in the maintenance of dorsoventral features of the leaf. In addition, they demonstrate the abaxial epidermis sends/receives a cell fate determining signal to/from the adaxial epidermis and controls the dorsoventral patterning of the maize leaf.

Key words: Maize, Leaf development, *Rolled leaf1* (*Rld1*), Dorsoventrality

INTRODUCTION

Plants, unlike animals, undergo a rhythmic development of lateral organs from a self-replenishing region of growth termed the meristem. One commonality between all lateral organs, for example leaves from a vegetative meristem or anthers from a floral meristem, is that they are polar from inception, displaying both proximodistal and dorsoventral (commonly referred to in plants as the abaxial/adaxial) axes.

The maize leaf and all other lateral organs develop from populations of 'segment' founder cells in the shoot apical meristem (Scanlon et al., 1996). Along the proximodistal (tip to base) leaf axis there are three readily visible regions of the maize leaf: proximal sheath, ligule/auricle region and distal blade (Fig. 1A). The dorsoventral axis contains five tissue layers (TL) that run continuously through the three regions of the proximodistal axis. The DV axis cuts through three tissue layer types: the outer epidermal layers (TL1 and TL5), the middle mesophyll and vascular layer (TL3), and the ground mesophyll layers (TL2 and TL4), as diagrammed in Fig. 6. The adaxial epidermis (TL1) and the abaxial epidermis (TL5) of the leaf have distinct epidermal cell type patterns (Freeling and Lane, 1993). The leaf's adaxial epidermis (TL1) can be distinguished by longitudinal files of macrohairs, which are large single cell hairs that occur in files of bulliform cells running parallel to the proximodistal axis of the blade. The adaxial surface of the leaf is also marked by the presence of ligule tissue at the blade sheath boundary. The abaxial epidermis (TL5) contains none of these features. The middle tissue of the leaf also shows dorsoventrality. Veins contain polarized xylem and phloem. Wild-type leaves also have a characteristic DV pattern of hypodermal schlerenchyma, which are specialized structural ground tissue cells associated with veins.

In recent years several new plant mutants have been reported to affect the DV leaf axis. Although it can be argued that the first of these mutants reported in dicots and monocots, *phantastica* and *leafbladeless*, are not true DV patterning mutants (Scanlon, 2000; Tsiantis et al., 1999), *Phabulosa*, *Kanadi*, and members of the YABBY family all seem to affect DV patterning (Kerstetter et al., 2001; McConnell and Barton, 1998; Siegfried et al., 1999). PHABULOSA is involved in specifying adaxial cell fate (McConnell et al., 2001), and *KANADI* and the *YABBY* gene family members are involved in abaxial cell fate determination (Kerstetter et al., 2001; Siegfried et al., 1999). *Rolled leaf1* mutants share some phenotypic similarities with the *Arabidopsis* mutants of *Phabulosa*, *kanadi*, and double YABBY family mutants.

In this paper we introduce and characterize the semi-dominant mutants of the maize *rolled leaf1* (*rld1*) gene. The allele we use most often is *Rld1-O*. The phenotypes of the group of mutant alleles are very similar in almost all aspects of the phenotype and we refer to this group of mutants as *Rld1* mutants. The characteristic tissue pattern associated with the maize leaf is abnormal and can broadly be defined as being misspecified along the dorsoventral axis. We will show that

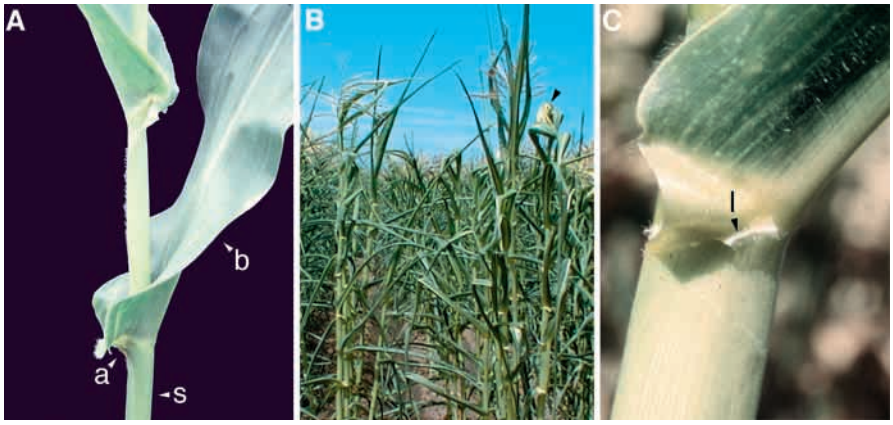

Fig. 1. Leaf phenotypes. (A) Wild-type blade (b), sheath (s), ligule/auricle region (a) indicated by arrows. (B) Field of *Rolled leaf1-0/+* mutants. The spiky appearance of leaves is caused by leaf blades being inwardly rolled, as opposed to lying flat, as seen in wild-type leaves in A. The leaf blades of *Rld1* mutants are often caught in the preceeding rolled-up blade and need to be unrolled in order to free the tassel (arrowhead). (C) Blade-sheath boundary of *Rolled leaf1* leaf, with an abaxial ligule (l).

Rld1 alleles act in one epidermis, but can switch the DV polarity of the entire leaf.

MATERIALS AND METHODS

Rolled leaf1 Genetic Stocks. *Rld1-O* ('O' for original) was recovered by Robert McBird and M. G. Neuffer from a population treated with MNNG (1-methyl-3-nitro-1-nitrosoguanadine) (Neuffer, 1990). *Rld1-1608* and *Rld1-1441* were also isolated by Bird and Neuffer, and recovered from populations treated with EMS (ethyl methanesulfonate). *Rld1-MF* and *Rld1-PB* were recovered from an undirected *Mutator* transposon mutagenesis screen in the Freeling Lab. These *Rolled leaf1* mutants were all introgressed into the inbred lines B73, Mo17, A637, W23 for several generations to obtain uniformity of characters not related to the Rolled leaf1 mutation within each introgressed family. We refer to this group of *Rolled leaf1* dominant mutants as 'alleles' because they share a particularly distinct mutant phenotype and map to the same locus (Chao and Neuffer, 1993; Lane and Freeling, 1996).

Microscopy

Autofluorescence

To examine internal leaf architecture, transverse hand-cut sections were observed on a Zeiss standard light microscope under epifluorescent illumination using a 395-440 nm excitation filter with a 470 nm long pass observation filter.

Staining of vascular bundles

Tissue from [P8]/[P9] (the 8th or 9th leaf away from the meristem) adult leaves were vacuum infiltrated for 20 minutes in a 3:1 ethanol/acetic acid fixitive and left at room temperature overnight. The tissue was then transferred into a 0.5% sodium metabisulfite solution for several hours. Subsequently the tissue was transferred into a diluted Schiff's reagent (0.1 % basic fuschin, 1.9% in 15:85 1N HCl/water) and shaken for several hours. 200 mg activated charcoal is then added to destain tissue. After Schiff staining, the tissue is returned to a sodium metabisulfite solution (0.5%) overnight. The tissue is dehydrated through an ethanol series followed by several changes of absolute ethanol. The samples are then placed in a Petri dish filled with ethanol and viewed using a dissecting microscope using methods adapted from Cheng (Cheng, 1995).

Epidermal impressions

Impressions were made to examine the epidermis of wild-type and *Rolled leaf1* mutant leaves. As suggested by L. Smith, UCSD, San Diego, USA, a drop of cyanoacrylate glue, (Quick Tite super glue) was placed on a glass slide and leaf tissue was pressed into it. Once the glue had dried, tissue samples were removed and slides were examined using (DIC) optics on a Zeiss standard light microscope.

Dosage analysis

The B-A translocation stocks, TB-9Lc and TB-9La, were obtained from the Maize Genetics Cooperative. *Rolled leaf1-O* and *Rolled leaf1-1608* heterozygotes were crossed as females by each B-A translocation stock. Our results from these two alleles were consistent, and pooled. Maize B chromosomes are supernumerary chromosomes that exhibit meiotic drive; the A chromosomes are the normal genetic complement of the maize genome. The B chromosome centromere often undergoes mitotic nondisjunction in the second microspore division resulting in sperm that are hyperploid and hypoploid for the A arm of the B-A translocation. This allows us to generate an aneuploid series, as the progeny of this cross segregate for genotypes *Rld1-O/–*, *Rld1-O/+*, *Rld1-O/++*, $rld1^{+/-}$, $rld1^{+/+}$, $rld1^{+/+}$+ where '+' is a 9L arm carrying a wild-type *rld1* allele and '–' is no arm 9L at all.

Mosaic sector analysis

A stock carrying the albino mutant *white luteus4-R* (*wlu4-R*), which maps approximately 45 map units proximal to *rld1* on chromosome 9L, was obtained from the Maize Genetics Coop. Plants heterozygous for *wlu4* were crossed onto heterozygous *Rolled leaf1-O* mutants. These crosses generated families segregating 1:1:1:1 for *Rld1-O* $Wlu4^{+}/rld1^{+}$ *wlu4*, $rld1^{+}$ $Wlu4^{+}/rld1^{+}$ *wlu4*, *Rld1-O* $Wlu4^{+}/rld1^{+}$ $Wlu4^{+}$ and $rld1^{+}$ $Wlu4^{+}/rld1^{+}$ $Wlu4^{+}$. Seven thousand five hundred seeds from these crosses were allowed to imbibe on paper towels for 24 hours at 30°C. From 50-100 seeds were transferred onto two moist Whatman filter papers in a 100 mm Petri dish. Imbibed seeds were irradiated with 1500 rads for approximately 7 minutes through a 0.21 mm Cu and 1.0 mm Al filters with a 160 kV Pantak X-ray machine run at 250 kV at Lawrence Berkeley Labs, Berkeley, CA. The irradiated seeds were planted in the summer nursery at the Gill Tract Field of UC Berkeley in Albany, CA.

Throughout development, plants were screened for albino sectors and sectored leaves were collected at maturity. These clonal sectors were presumed to be aneuploid for the albino marker (*wlu4/–*). Examination of nonmutant sibs ($rld1^{+}$ $Wlu4^{+}/rld1^{+}$ *wlu4)* showed that the partial loss of chromosome 9L did not affect the morphology of the mature leaf. A total of 32 *Rld1-O* plants were found to contain sectors and were examined for the Rolled leaf1 phenotype in and around the sector. Leaf number, sector width and position, color and shape were recorded for each sector and sectors were drawn schematically. Transverse sections of these leaves were made by hand. Observations were made on a Zeiss standard light microscope under epifluorescent illumination, using a 395-440 nm excitation filter with a 470 nm long pass observation filter as described previously (Becraft and Freeling, 1994). Using these conditions, normal green

chloroplasts fluoresce red and chloroplasts in albino tissue fluoresce a light yellow. All mesophyll layer cells contain chloroplasts. The genotype of the epidermal layers were determined by examining the chlorplast-containing guard cells. Since chromosome loss can occur in varying tissue layers, we recorded the phenotype within and near white sectors and which tissue layers (TL1-TL5) were affected. Blade sectors were scored for the presence and position of epidermal macrohairs and hypodermal schlerenchyma. Juvenile leaves were not analyzed because they lack epidermal macrohairs. White sectors and surrounding tissue layers were scored using these phenotypes as being either *Rolled leaf1* or non mutant.

RESULTS

Expression of dorsoventrality in the wild-type maize leaf

As is typical of many grasses, adult maize leaves exhibit a number of distinct epidermal cell types (Fig. 2A). The leaf surface consists of long epidermal cells with undulate walls organized into a two-dimensional pattern with stomata, trichomes, and silica cells (Esau, 1977). There are three types of trichomes present: microhairs, prickle hairs and macrohairs. The latter two types of hairs are associated with rows of bulliform cells, enlarged cells involved in controlling curvature of the leaf in response to water stress (Esau, 1977). A pair of narrow guard cells and a flanking pair of subsidiary cells together make up a stomatal complex, large numbers of which are spaced regularly on the epidermal surfaces. These different cell types are found in an orderly pattern and allow us to easily differentiate between the upper (adaxial, dorsal) epidermis of the leaf and the lower (abaxial, ventral) epidermis of the leaf. For example, the density of stomatal complexes differs on the two surfaces. An obvious distinguishing characteristic is that macrohairs are seen only on the adaxial epidermis (in bulliform cell rows) and are completely absent on the lower epidermis (Fig. 2B,D). The adaxial surface of the leaf is also marked by the presence of ligule tissue at the blade sheath boundary.

Effects of *Rolled leaf1* mutants on leaf curling and epidermal switching

The phenotypes imparted by the different *Rolled leaf1* mutant alleles (*Rld1-O, Rld1-1608, Rld1-1441, Rld1-MF, Rld1-PB)* closely resemble one another (data not shown). All mutant plants are identified at the gross morphological level by a straighter longitudinal habit and a transverse curvature of the leaf blade with the lamina rolling inward towards the adaxial midvein (Fig. 1B). This transverse rolling of the leaves is characteristic of maize plants suffering from water stress. However, Hay and coworkers (Hay et al., 2000) examined the biomechanics behind this curvature and concluded that the rolling was not the consequence of any loss of turgor and furthermore the straightened longitudinal shape of the leaves was independent of the transverse rolling. *Rld1* mutant leaves also show a number of defects in the DV axis. They display a partial to complete switching of the epidermal surfaces. *Rld1* blades are characterized by macrohairs on the abaxial epidermis (Fig. 2E). This adaxialization of the abaxial epidermis is usually associated with the loss of macrohairs on the adaxial epidermis (Fig. 2C). Such cases constitute obvious switches in epidermal identities. Yet, frequently, areas are observed where both abaxial and adaxial epidermal surfaces have adaxial characters giving the tissue adaxial/adaxial (ad/ad) polarity. Usually, both boundaries of switched regions with abaxial/adaxial polarity show adaxial/adaxial polarity (data not shown).

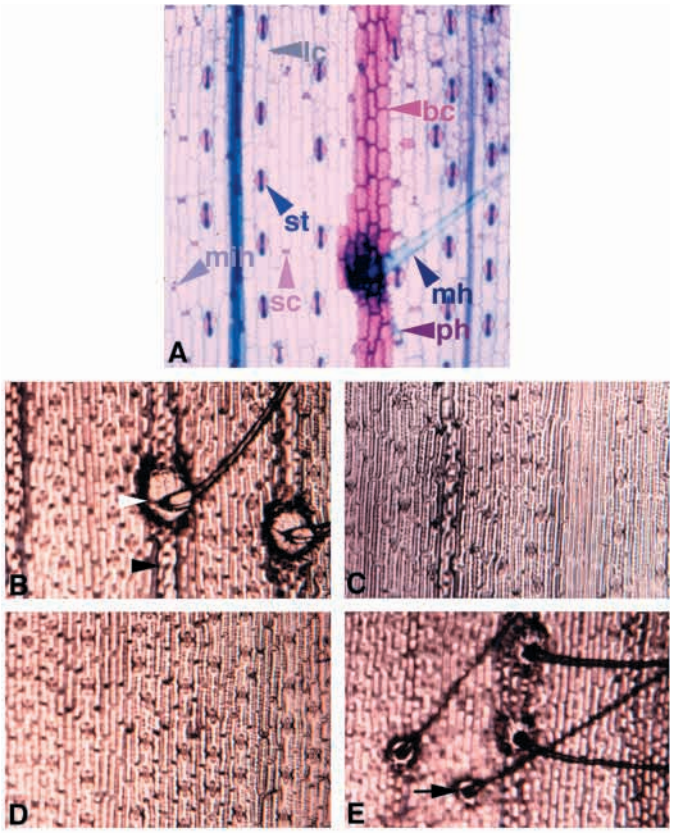

Fig. 2. Epidermal peel of wild-type adaxial blade and impressions of wild-type and mutant blade epidermises. (A) Wild-type epidermal tissue is characterized by a very regular pattern of specialized cells: macrohairs (mh), pricklehairs (ph), microhairs (mih), bulliform cells (bc), long cells (lc), silica cells (sc), and guard cells with associated subsidiary cells, which together make up stomatal complexes (st). (B) Macrohairs (white arrowhead) and bulliform cell rows (black arrowhead) are seen in the wild-type adaxial epidermis. In contrast, no macrohairs are seen in the wild-type abaxial epidermis (D). (C) Adaxial epidermis of *Rolled leaf1-PB* heterozygous mutant. (E) Abaxial epidermis of *Rolled leaf1-PB* heterozygous mutant. Arrow indicates macrohair.

Abaxial ligule flap and irregular venation patterning

The blade-sheath boundary of wild-type leaves is marked by the presence of a ligule. The ligule is continuous along the adaxial surface from margin to margin. All *Rolled leaf1* mutant alleles result in leaves with an abaxial ligule flap in addition to the adaxial ligule (Fig. 1C). That is, the ligule domain at the blade/sheath boundary in *Rld1* mutants is adaxialized on both epidermal surfaces and never displays the switching seen with the macrohairs. The expressivity of this abaxial ligule flap phene changes from plant to plant and leaf to leaf of the same plant. The abaxial ligule flap varies in width, sometimes extending from margin to margin and other times being just a few millimeters in width. When the abaxial ligule flap does not

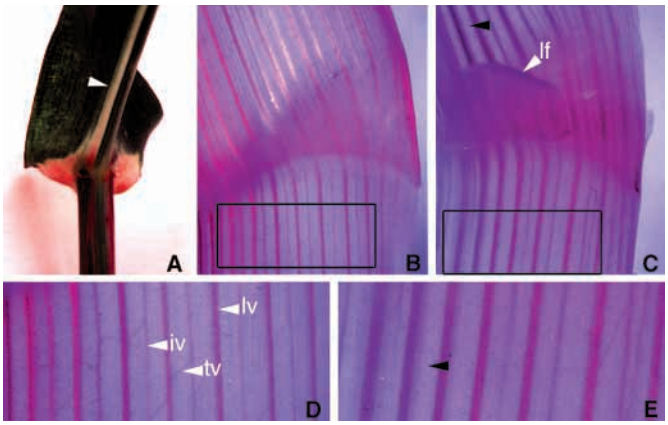

Fig. 3. Schiff staining of 'clearing' at the blade sheath boundary. (A) *Rolled leaf1-PB* mutant with region of pale tissue indicated by an arrowhead. (B) Abaxial view of veins at the blade-sheath boundary of a wild-type leaf, as observed with Schiff staining. (C) Abaxial view of blade-sheath boundary in a *Rld1* leaf at region of pale tissue (arrowhead in A), located in same longitudinal region as the ligule flap (lf). Schiff-stained vasculature in *Rld1-PB* leaf shows lack of intermediate and transverse veins (black arrowhead) in this 'clearing.' (D) Enlarged view of boxed area in B, showing intermediate veins (iv) and transverse veins (tv) between the lateral veins (lv). (E) Enlarged view of boxed area in C showing lack of intermediate and transverse veins in the region of the 'clearing' (black arrowhead).

extend across the entire width of the blade, the flap becomes a pair of flaps placed symmetrically on either side of the midrib.

Proximal and distal to the pair of ectopic ligule flaps, *Rolled leaf1* mutant leaves display varying degrees of pale tissue extending from the ligule flap into both the blade and sheath (Fig. 3A). The ground tissue cells in these pale sectors were not green, suggesting absence of chloroplasts or chlorophyll. We compared wild-type vasculature with mutant *Rolled leaf1* leaf vasculature. Wild-type leaf blade and sheath display a regular parallel pattern of lateral veins separated by many intermediate veins in the blade and at least one intermediate vein in the sheath (Fig. 3B,D). In addition, there are transverse vascular bundles running horizontally between the lateral and intermediate veins (Fig. 3B,D). The pale tissue or clearing found in mutant *Rolled leaf1* leaves is associated with lack of development of intermediate and transverse vascular bundles in that region (Fig. 3C,E).

Rld1 affects sub-epidermal tissue

Hypodermal schlerenchyma associated with intermediate veins in the lamina of the wild-type leaf are predominantly associated with the abaxialepidermis (Fig. 4A). In the regions of the lamina undergoing dorsoventral switching in *Rolled leaf1-MF* mutants, this bias is switched. The hypodermal schlerenchyma in these areas of *Rolled leaf1* mutants are more often associated with the adaxial epidermis (Fig. 4B).

Dosage analysis shows that mutant *Rld1* acts antagonistically to the wild-type gene

Owing to the complexity of the function of a wild-type allele from a dominant mutant phenotype, we wanted to determine the nature of the dominant mutation. Based on the result that,

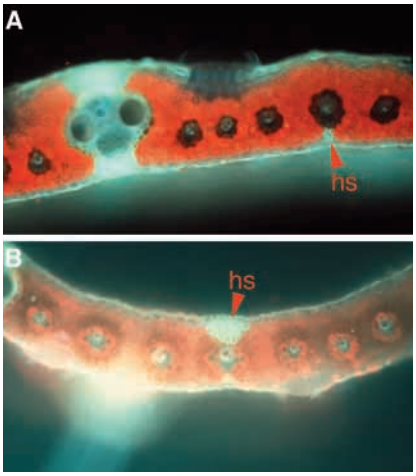

Fig. 4. Subepidermal architecture. Hypodermal schlerenchyma (hs) in wild-type blade tissue is more frequently associated with the abaxial epidermis (A). In regions of *Rolled leaf1* mutants where the epidermal characters have been reversed, hypodermal schlerenchyma is frequently seen on the other side near the adaxial epidermis (B).

in some cases, the mutant allele is able to completely switch both epidermal surfaces, we suspected that the *Rolled leaf1* mutants were not going to be neomorphic mutants. A dosage analysis experiment was used to test this hypothesis. Using the B-A translocation system we were able to vary the dosage of the long arm of chromosome 9. We generated an aneuploid series for *Rld1-O* and rld^{+}: the family segregated for genotypes *Rld1-O/–*, *Rld1-O/+*, *Rld1-O/++*, $rld1^{+/-}$, $rld1^{+/+}$, $rld1^{+/+}$+ where '+' is a 9L arm carrying a wild-type *rld1* allele and '–' is no arm 9L at all. Fig. 5 shows a comparison of the leaf at the node above the primary ear for plants of the genotype *Rld1-O/–*, *Rld1-O/+*, and *Rld1-O/++* (hypoploids, euploids and hyperploids respectively). Total numbers of plants carrying one dose of *Rld1-O* were 4 hyperploids, 6 euploids, and 5 hypoploids representing 2, 1 and 0 wild-type alleles added respectively. Of the three genotypic classes, the hyperploids

Fig. 5. Leaf phenotypes resulting from dosage series. Aneuploidy experiments showing three sibling leaves with one copy of *Rolled leaf1-O*, but differing in dosage of the wild-type allele, $rld1^{+}$: (left) *Rld1-O/*$rld1^{+}rld1^{+}$(hyperploid), (middle) *Rld1-O/*$rld1^{+}$ (euploid), (right) *Rld1-O/–* (hypoploid). *Rld1-O/*$rld1^{+}rld1^{+}$ exhibits the least severe mutant phenotype. The abaxial ligule (black arrowhead) is present in only a narrow portion (as shown by size of black bar above ligule) of the blade width and the vascular disturbance is mild resulting in negligible 'clearings' (red arrowheads). Clearings result from a disturbed pattern of venation in the mutant (Fig. 3E). The width of the blade lamina is very similar to that observed in wild-type siblings for the same dosage series (data not shown). As $rld1^{+}$ alleles change in dose from 2 to 0, all components of Rld1 phenes become more severe as can be seen for leaf-width and clearings.

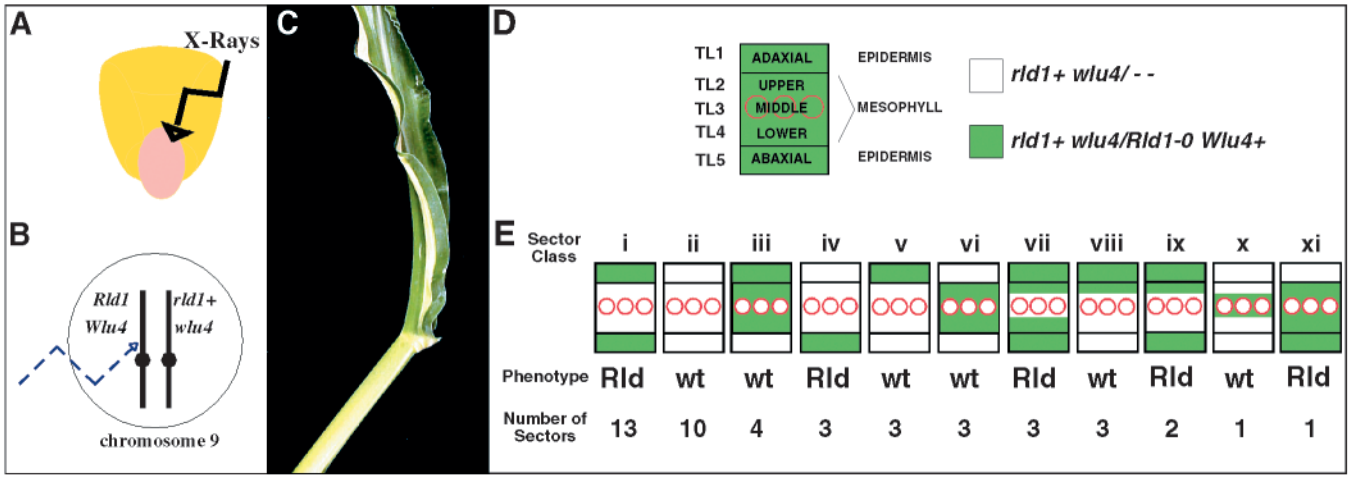

Fig. 6. Construction and phenotypes of genetic mosaics. (A) Seeds were X-irradiated to generate mosaic plants. (B) Cartoon of a cell showing a pair of chromosome 9 and relative positions of alleles of *rld1* and *wlu4*. The arrow indicates an X-ray breakage event. The somatic loss of the 9L arm will result in a lineage of cells mutant for *wlu4* (white) and no longer carrying the mutant *Rld1-O* allele. (C) Mature leaf from plant heterozygous for *Rld1-O* and *wlu4* (phenotype is Rolled leaf1 and green) with a white sector (hemizygous for *rld1*$^{+}$*wlu4*). (D) Schematic showing cross section of a leaf (DV axis) with the five layers (TL1-TL5), and tissue types indicated. (E) Cartoons representing the different classes (i to xi) of leaf sectors found in X-irradiated *Rolled leaf1-O* plants, white indicates the *rld1*$^{+}$*wlu4* genotype and green indicates the *Rld1-OWlu4*$^{+}$ genotype. The resulting phenotype (wild-type (wt) or Rolled leaf1(Rld1)) of each of the sectors and the total numbers for each sector class are shown. Sectors within each class came from different chromosome breakage events.

were the plants with the narrowest abaxial ligule and the mildest vascular disturbance. In *Rld1-O*/++ plants, the intermediate and transverse veins were missing in only a small region of the sheath, just below the ligule. In the euploids, the region with missing vasculature extended across as a larger width of the sheath and farther below the ligule than in the hyperploids and also extended distally into the blade region. The hypoploids had the most severe mutant phenotype. The region of vascular disturbance extended over a greater width of the blade, and extended farther longitudinally, away from the ligule, than in either of the other genotypes. The hypoploids also had the narrowest leaves. These data suggest that the severity of the *Rld1* phenotype decreased as copies of the wild-type allele are added. Control plants segregating this aneuploid series but not carrying *Rolled leaf1-O* do not account for these differences (data not shown for 11 control plants). These results suggest that *Rolled leaf1-O* mutants act antagonistically to the wild-type *rld1* gene. Similar results were obtained for a dosage analysis with the *Rld1-1608* allele (data not shown).

Mosaic analysis

A mosaic analysis was done to identify the focus of action of the mutant gene product. The experiment was designed to expose recessive alleles by X-ray induced chromosome breakage using a linked gene which is visible and cell autonomous to mark the sector (Hake and Freeling, 1986) (Fig. 6A-C). We created a total of 32 white sectors (*rld1*$^{+}$*wlu4*/– –) within mutant plants. This yielded 46 periclinal chimeras, which were put into 12 classes (Fig. 6E). We scored the phenotype within and near these white sectors. For the majority of the sectors, *Rld1-O* was lost in only the internal layers, TL2, TL3, and TL4 (class i); and the resulting phenotype of such sectors was Rolled leaf1. The next most common sector type found is a loss of *Rld1-O* in all tissue layers (class ii) and its phenotype was nonmutant. The most informative sectors were of two 'reciprocal' classes. First, when *Rld1-O* is present in only the abaxial epidermis (class iv), the sector is of *Rolled leaf1* mutant phenotype (Fig. 6E, Fig. 7D,F,H). This includes mutant phenes in other layers along the dorsoventral axis (mesophyll, TL2 and TL4) and adaxial epidermis (TL1). The second class consisted of sectors where *Rld1-O* is absent in only the abaxial epidermis (class iii); these sectors are phenotypically wild type (Fig. 6E, Fig. 7C,E,G). All other classes of sectors supported these findings (classes v-xi). These results indicate that *Rld1-O* in the abaxial epidermal cell layer is both necessary and sufficient in our genetic backgrounds for the expression of the Rolled leaf1 phenotype throughout the leaf.

Two issues regarding our sectors need to be addressed. First, sectors in which the ground tissue (TL2, TL3, TL4) was green surrounded by one or more white epidermal layers (TL1 and/or TL5), which are undetectable by the eye in the field, were observed at the margins of sectors that were easily identified by their pale green or white nature. These sectors were 'shelves' adjacent to these pale green or white sectors and were derived from the same single cell event. Therefore chromosome breakage in a single cell produced different types of periclinal chimeras, providing data for multiple sector classes as noted in Fig. 6E. Second, sector size is an indicator of the developmental time at which the *Rld1-O* was lost. A large distribution of sector sizes was recovered indicating our results were consistent over a window of developmental time.

All aspects of the *Rolled leaf1* phenotype are non cell-autonomous in the dorsoventral dimension. We could not definitively measure cell autonomy of the *Rolled leaf1* phenotype in the lateral dimension because the markers used are macrohairs and hypodermal schlerenchyma. These markers occur periodically across the lateral dimension and sector boundaries did not always coincide with these markers. Therefore, we cannot eliminate the possibility of lateral

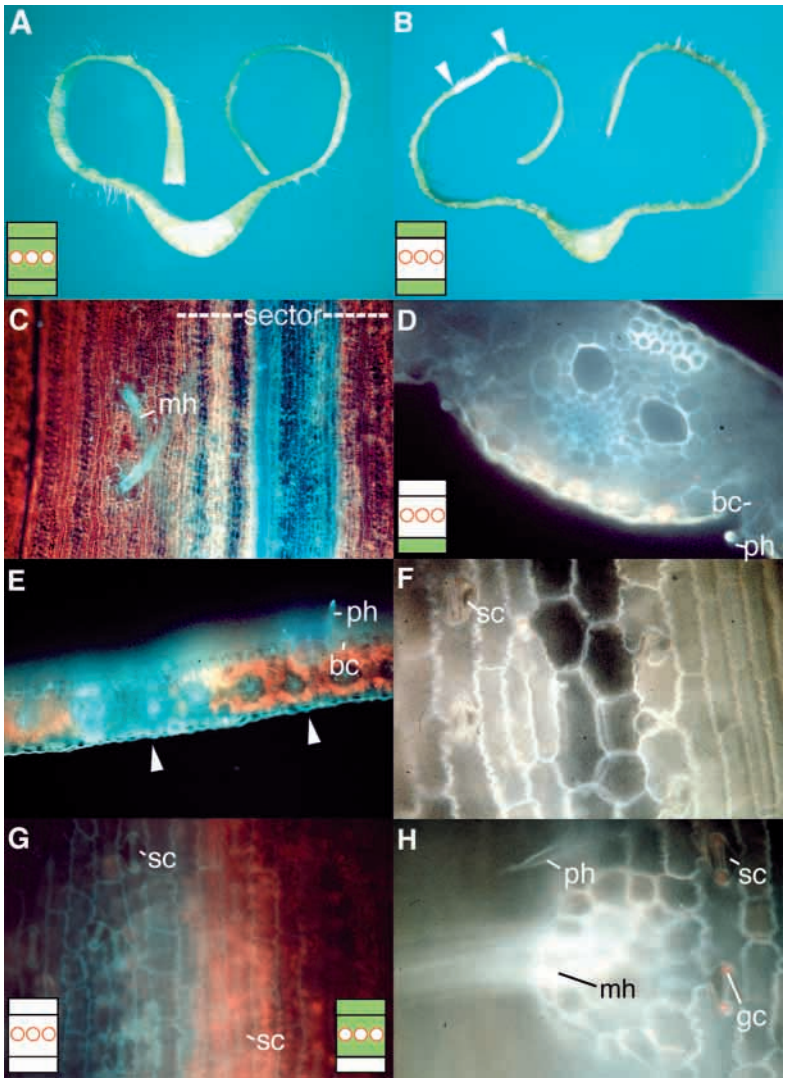

Fig. 7. Leaf phenotypes of sectors from mosaic analysis. Transverse sections of Rolled leaf1 leaves containing non mutant white sectors. Plants are genotypically *rld1*$^{+}$*wlu4/Rld1-OWlu4*$^{+}$ (*Rolled leaf1* phenotype, green tissue). White tissue indicates removal of dominant mutant allele *Rld1-O* from particular tissue layers of sector. (A) Leaf with no white sectors as shown by inset cartoon. Macrohairs on the abaxial epidermis show the characteristic Rolled leaf1 phenotype. (B) White sector, indicated between arrowheads, marks the loss of *Rld1-O*. Close examination showed epidermal guard cells were still green, as shown by inset. This sector had the typical *Rld1-O* polarity as the presence of macrohairs on the abaxial epidermis in white tissue sector indicate. (C) Micrograph of abaxial epidermis with a sector running through it. To the left of the sector there are macrohairs (mh). No macrohairs are observed on this epidermis in the sector. (D) Transverse section of a sector where *Rld1-O* has been lost in all tissue layers except the abaxial epidermis (TL5) as shown by inset. Prickle hairs (ph) and bulliform cells (bc) are present on the abaxial epidermis. (E) Transverse section of a region of the sector seen in C. Prickle hairs and bulliform cells are present on the adaxial epidermis. (F) Adaxial epidermis of Rld1 sector seen in D, where *Rld1-O* was removed in all but the abaxial epidermis. Stomatal complexes (sc) contain guard cells that are white. (G) Abaxial surface of the region of sector in E marked by arrowheads. *Rld1-O* has been lost on this epidermis as observed in the stomatal complexes that are not fluorescing red. Inset cartoons show genotypes of each half of this sector. Tissue is phenotypically wild type. (H) Abaxial epidermis of same sector as F. Chloroplasts of guard cells (gc) are fluorescent red indicating presence of *Rld1-O*. The presence of macrohairs and prickle hairs on the abaxial surface indicate the sector is phenotypically Rolled leaf1.

signaling that extends for a few cells. However we never saw the *Rolled leaf1* phenotype above wild-type abaxial epidermis.

DISCUSSION

The *Rolled leaf1* mutants display variability and complexity in their expression of phenes. Taking into consideration what is already known about the normal process of development of both the organ and plant, we have analyzed how the disruption of a single gene could create this variety of phenes and this variability of expression. One explanation for the mutant phenotype implicates the *Rld1* gene product's involvement in maintaining tissue polarity along the dorsoventral axis.

Rld1 acts after initial dorsoventrality is established

Rld1 leaves display a partial DV switching of epidermal tissue and ground tissue with a bias towards adaxialization. Polarity appears normal over regions of varying width across the leaf. The affected area may be localized between only two lateral veins or may include several lateral veins. In regions with altered polarity, the tendency is toward adaxial/abaxial (ad/ad) polarity. However, many *Rld1* mutants have regions where the epidermal and sub-epidermal tissues have been flipped, giving abaxial/adaxial (ab/ad) polarity to the tissues (Fig. 8). Hay et al. (Hay et al., 2000) found that abaxial fiber cell density was greater in wild-type than in *Rld1* mutant leaves, supporting the hypothesis that differential fiber development between the two surfaces produces curvature. Our observations also support the theory that differential schlerenchyma distribution leads to the inward rolling of the leaf. The fact that mutant leaves exhibit dorsoventrality and have normal vascular polarity at inception indicates that *rld1+* is not involved in setting up initial dorsoventral polarity but rather in the reception, propagation, or maintenance of this state (Fig. 8).

In *Rld1* mutants, intermediate and transverse veins fail to elaborate within leaf tissues with altered dorsoventral polarity. In wild type, primary intermediate veins develop basipetally at the leaf margins from lateral veins (which had developed acropetally) and as the leaf primordium widens, these primary intermediate veins branch to form a network of basipetally differentiating intermediate veins (Sharman, 1942). Transverse veins differentiate last, forming a net among the longitudinal veins of the leaf (Sharman, 1942). The procambial strands, that

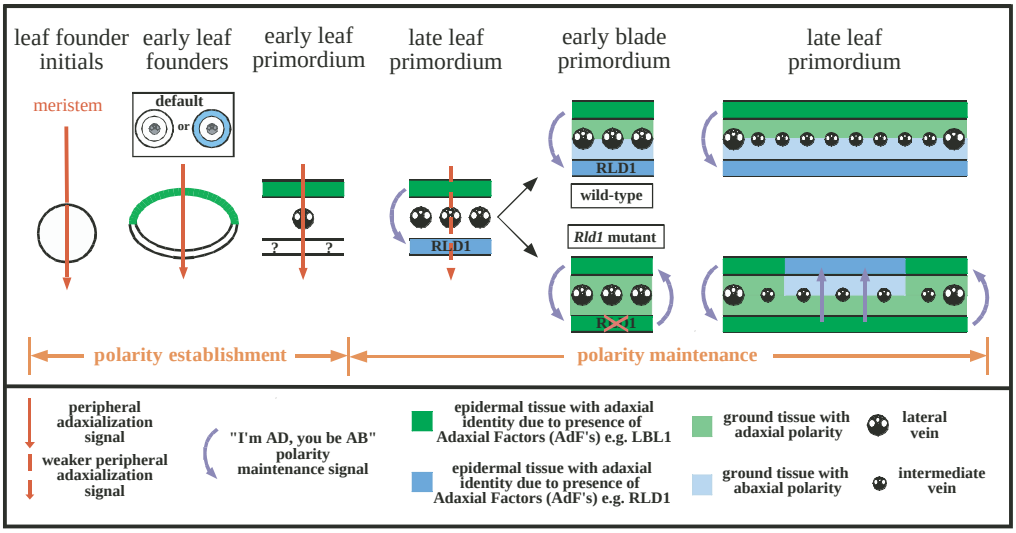

Fig. 8. One model that explains how RLD1 normally functions in the abaxial epidermis to promote adaxial identity. In this model, dorsoventrality is initiated by a peripheral signal and RLD1 is involved in its maintenance. At P0, the leaf founder initials lack polarity. As these initials develop into the early leaf founder cells, they develop polarity in response to a peripheral/adaxialization signal emanating from the meristem. The surface closest to the meristem takes on adaxial identity and the surface further from the meristem might have abaxial identity as a default or abaxial identity might be specified on tissue with undefined cell fate (see inset) as a result of a concentration differential or via a signal cascade started by the peripheral signal. By the late leaf primordium, as the developing primordium is moving further away from the meristem, the peripheral signal is no longer a strong enough influence on the primordia and another mechanism for polarity maintenance is necessary. One possible model for polarity maintenance is a polarity maintenance signal (PMS) emanating from tissue with adaxial identity (light blue arrow). This reinforces its adaxial identity via adaxial factors (AdFs) such as LEAFBLADELESS1 (LBL1), and signals to the opposite half of the leaf to maintain abaxial identity. Abaxial factors (AbFs) such as ROLLED LEAF1 (RLD1) are responsible for maintenance of abaxial polarity. As the blade primordium develops, this 'I'm AD, you be AB maintenance signal remains important to maintain correct polarity. In *Rld1* mutants, the mutant RLD1 interferes with the function of wild-type RLD1. The mutant AbF is unable to maintain abaxial identity in the abaxial tissue and results in a blade phenotype of abaxial tissue being switched to adaxial identity as a mutant response to either the weaker peripheral/adaxialization signal or to the PMS from the adaxial tissue. The lower tissue having taken on adaxial identity, now sends out the PMS to the upper surface, and sometimes in *Rld1* mutants, some part of this upper surface (adaxial tissue) responds to the signal, becoming abaxialized. This results in the dorsoventral axis being flipped over in some portion of a *Rld1* mutant leaf. However, an alternative model where the abaxial epidermis generates an 'I'm ab so you are ad' trans-tissue signal is equally likely (not shown).

give rise to mature veins, develop in continuity from previously formed procambium (Pray, 1955). The missing intermediate and transverse veins in the 'clearings' of *Rld1*mutants might be explained by failure to develop the veins from tissue with normal ad/ab polarity into tissue that has been mis-specified with ad/ad or ab/ad polarity. This disturbance of the vascular patterns suggests that correct polarity information from the surrounding tissues is required for expansion of the vascular network. Perhaps pre-existing vein polarity is propagated through the process of branching and vein extension. Whether this process is actually dependent on correct polarity being maintained or whether it is the consequence of a secondary function, or malfunction, of the *rld1+* product remains to be determined. The green bundle-sheath cells in C4 grasses are products of the vascular lineage (Langdale et al., 1989). With the loss of veins observed in the mutant, there is a loss of color in this tissue associated with the loss of photosynthetic bundle sheath. The fact that the mesophyll tissue is not green in these 'clearing' suggests that these cells might have been arrested as immature ground tissue precursor cells. Perhaps, there is a requirement of vein development and lateral growth for ground tissue differentiation. Alternatively, the polarity of these sectors is so disrupted that the tissue neither differentiates nor develops the normal compliment of veins.

Dosage and mosaic analyses suggest *rld1+* functions in the abaxial epidermis

To gain insight into how the dominant *Rld1* mutant allele functions in relation to the wild-type allele, we performed a dosage analysis. Dosage analysis determines the effect of changing copies of *rld1*$^{+}$, in a mutant *Rld1* plant. The results of our dosage analysis indicate that addition of *rld1*$^{+}$ alleles to a *Rld1-O* mutant allele reduces the severity of the phenotype. This result suggests that *Rld1-O* is an antimorph, or 'dominant negative' mutation. Antimorphic dominants are interpreted as mutants that encode a product that antagonizes normal gene activity (Muller, 1932; Poethig, 1988). Since an antimorph acts by inhibiting the wild-type allele's function, it follows that tissues affected by the mutant phenotype are within the domain of RLD1 function.

The mosaic analysis was conducted on *Rld1-O* mutants to determine if the phenotype is cell-autonomous or non cell-autonomous and to identify the focus of action of the dominant mutant gene product. Our results indicate that a signal emanating from the mutant abaxial epidermis is able to affect all five tissue layers. This includes altering the characteristic cell types of both epidermal layers (TL1 and TL5), changing the polarity of the hypodermal schlerenchyma in the subepidermal mesophyll layers (TL2 and TL4), and disturbing the formation of intermediate and transverse veins in the middle mesophyll layer (TL3), but this was not closely scored in the mosaic analysis. It is interesting that such a pleiotropic mutant phenotype is caused by a disturbance in a gene that acts within a single cell layer.

rld1^{+} acts in the abaxial epidermis and is involved in trans-epidermal signaling that reinforces polarity

The data from the mosaic analysis taken with our results from the dosage analysis imply that *rld1*$^{+}$ acts in the abaxial epidermis. Our dosage analysis showed that, in *Rolled leaf1*

mutants, the mutant *Rld1* product is competing with the wild-type RLD1. The antimorphic data from the dosage analysis allows us to interpret *Rld1* like a loss-of-function mutant. Our mosaic analysis showed the focus of the mutant *Rld1* to be the abaxial epidermis. It follows that *rld1*$^+$ is involved in initiation or maintenance of the abaxial epidermal identity and in propagation or maintenance of the correct polarity of the leaf's DV axis. Furthermore, we never find abaxial/ abaxial polarity in *Rld1* mutants, supporting our conclusion that *rld1*$^+$ promotes abaxial identity in the lower epidermis.

The *Rld1* mutants also give us a unique insight into the complexity of signaling involved in setting up the correct DV polarity. Our results suggest a transverse signaling mechanism from abaxial epidermis to adaxial epidermis, affecting the polarity of schlerenchyma and veins. The abaxial surface of *Rld1* mutant leaves is partially adaxialized and often this is accompanied by the adaxial epidermal tissue directly above being abaxialized. This reversal of epidermal tissue suggests there is communication between the two surfaces, perhaps a polarity maintenance signal (PMS) from one epidermis to the other, reinforcing the other epidermis to be the opposite identity to maintain correct dorsoventral polarity (Fig. 8). It is still undetermined if this signal emanates from the adaxial surface to reinforce abaxial identity or if it emanates from the abaxial surface and reinforces adaxial identity. Yet, switching has never been seen in the ligular region and sometimes does not occur in the blade, thus leading to two adaxial surfaces which are most commonly observed at the boundaries of a switched, ab/ ad, region. However, sometimes homogenous regions of ad/ab polarity exist. Hence our mutant phenotype suggests a two step process for polarity maintenance. In *Rld1* mutants first there is mis-specification of the abaxial epidermis to adaxial, and secondly, in some cases, there is reversal of the adaxial epidermis to abaxial identity. Since we do not always see step two (epidermal switching), we hypothesize that timing does not always permit this to occur. The cells of the adaxial epidermis might become determined to that fate and are no longer responsive to the signal from the lower epidermis that has taken on adaxial tissue identity (Fig. 8) or there is a leaky signal still coming from the abaxial epidermis maintaining adaxial tissue identity in the upper tissue layers (not shown). Therefore other factors must influence some part of the *Rld1* trans-tissue signaling pathway.

One model for initiation and maintenance of dorsoventral polarity involves three separate steps. Based on the results of surgical experiments suggesting that a factor, a peripheral signal, emanating from the center of the shoot apex is necessary for development of the adaxial domain (Hanawa, 1961; Snow and Snow, 1959; Sussex, 1954; Sussex, 1955), the first step in polarity establishment is dependent on a morphogen, the peripheral/adaxialization signal (PAS), coming from the meristem (the red arrow in Fig. 8). Adaxial factors (AdFs) in the surface of the leaf primordia closest to the meristem are responsible for perception and propagation of this signal to form a flattened leaf and adaxial tissue identity. It is unknown whether the default state is a radial leaf with abaxial identity or if both abaxial and adaxial identities are independently specified through AdFs and abaxial factors (AbFs). If abaxial identity is not the default state of the leaf, it might be established as a response to lower morphogen concentrations or via a signal cascade initiated by the PAS, both of which might involve AbFs. As the developing leaf grows away from the meristem, the source of the PAS, a new mechanism is needed for maintenance of dorsoventral polarity.

Regardless of whether abaxial and adaxial tissues are independently specified, our data proves that they interact in the maintenance of their identities. This second step involves feedback from either of the leaf surfaces to the other in the form of a polarity maintenance signal (PMS): 'I'm adaxial, you be abaxial' trans-tissue PMS (Fig. 8) (or 'I am abaxial, you be adaxial' trans-tissue PMS, not shown). RLD1 is involved in the maintenance of the DV axis. RLD1 might be responding to the PMS which signals the abaxial half of the blade primordium to maintain abaxial identity. Alternatively, RLD1 might interact directly or indirectly (via AdFs) with the PAS and inhibit its action in the abaxial epidermis, preventing adaxialization of the lower surface of the leaf . In *Rld1* mutants this step results in two adaxial surfaces. Additional genetic support for step two and our interpretation that RLD1 normally abaxializes the most peripheral epidermis comes from double mutants between *Rld1* and *leafbladeless1* (*lbl1). leafbladeless1* mutant plants are abaxialized. Juarez and Timmermans (Juarez and Timmermans, 2001) reported that the double mutant resulted in mutual suppression of both phenotypes as expected if *Rld1* adaxialized and *lbl1* homozygote abaxialized. However, our data did not show mutual suppression, but did show that *Rld1-1441* almost completely suppressed the *lbl1-R* phenotype whilst the *Rld1-1441* phenotype remained strong (J. M. N. and M. F., unpublished).

Our results suggest that sometimes the developmental window still allows the adaxial half of the blade primordia to become abaxialized, suggesting a third step. This third step involves further reinforcement of dorsoventral identity in the developing blade which is no longer receiving any PAS morphogen from the meristem (Fig. 8, late blade primordium). In *Rld1* mutants, this switch might either be due to tissue expressing AdF's signaling the opposite side to express abaxial characters, which in the mutant case can not affect the abaxial surface but can affect the adaxial surface (Fig. 8). Or the switch might result from reversion of the adaxial tissue to a default abaxial identity, if the PMS emanating from the abaxial epidermis relies on AbFs which are not expressed in the *Rld1* mutant (not shown).

Regarding the developmental time at which of RLD1 acts, we believe our model is supported by the fact that the polarity of the vasculature of Rld1 leaves is normal. This suggests that early in leaf development, during the acropetal and the basipetal waves of vein differentiation, the polarity of the leaf is normal in Rld1 mutants. Based on phenotype and antimorphic data, we propose RLD normally acts following this stage during the differentiation of epidermal-specific and subepidermal-specific cell types which follows vein differentiation.

The occurrence of homogenous ad/ad leaf regions is central to our three-step model for achieving a switched region. It is also correct that most ad/ad regions occur at the boundaries of a switched region. One way to account for these data is if the initial event is a regional switch from ad/ab to ab/ad, and then (step two) for there to be a lateral signal where adaxial identity is propagated. The result would be ad/ad borders around the switched region. If these borders extend and run-together, a homogenous region of ad/ad leaf might occur indirectly (see

Acknowledgements). Indeed, the signaling details that cause the switch in dorsoventrality are unknown.

If location is an indicator of function, the YABBY genes are like ROLLED1. The expression of YABBY genes in *Arabidopsis* is polarized to the abaxial half of the lateral organ (Siegfried et al., 1999). Ectopic expression phenotypes of *Filamentous Flower* (*Fil*) and *Yabby3* (*Yab3*) confer abaxial cell identities onto adaxial surfaces of leaves (Siegfried et al., 1999). Mutants in *yab3* and *fil* that reduce or eliminate function respectively, have no phenotype (Siegfried et al., 1999; Sawa et al., 1999; Chen et al., 1999). However, the *fil yab3* double mutant has a phenotype which has been interpreted as partial adaxialization of abaxial cell types (Siegfried et al., 1999). This observation suggests that there is functional redundancy within this gene family. This double mutant phenotype resembles that of *Rld1* mutants in its partial adaxialization of abaxial tissue. However, it is important to point out that the *Rld1* mutants phenotype remains unique in being the only dorsoventral polarity mutant phenotype in which switch in the polarity of the tissue has been observed.

With the existing data on YABBY genes and *Rld1*, there is no obvious way to fit *Rld1* into the *Arabidopsis* dorsoventrality network. However, we will speculate on a few possibilities. One explanation of *Rld1*dominant mutants is that they down-regulate more than one YABBY gene, either directly (e.g. at the transcription level) or indirectly. This speculation is attractive because dominant negative mutants have the ability to remove a family of gene products from a specific time and place in development. Alternatively, RLD1 function could be necessary for any YABBY function, either upstream or down, or in balances of YABBY function. Siegfried and others (Siegfried et al., 1999) proposed that relative expression patterns of abaxial and adaxial specific genes might be responsible for differentiation of epidermal identities. Our results prove that there is communication between the epidermal layers, but the mechanism remains to be determined. We should emphasize these ideas are just conjecture because they require a level of regulatory homology between maize (a monocot) and *Arabidopsis* (a dicot) for which there is no evidence.

Mutations in the *rld1* gene, like mutations in most of the genes involved in establishing dorsoventral polarity in leaves and other lateral organs, do not result in a complete loss of polarity. Our results suggest that there might be some redundancy in the maintenance of dorsoventral polarity in maize. We have found a dominant mutant mapping to a new gene, *Rolled leaf2* (*Rld2)*, which specifies an indistinguishable phenotype from the *Rld1*.

Our model suggests wild-type RLD1 is an abaxial factor required for maintenance and perhaps reception or propagation of abaxial tissue identity. The *Rolled leaf1* mutant is unique in uncovering a signal transduced across the transverse dimension of the leaf, which is responsible for maintaining dorsoventral polarity in the leaf. It is possible that *rld1*$^{+}$ functions in a dorsoventral signaling pathway that operates within the leaf only. We conclude that RLD1 is a novel component of the DV patterning signal network.

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