Mosaic analysis of the dominant mutant, *Gnarley1-R*, reveals distinct lateral and transverse signaling pathways during maize leaf development

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**SUMMARY**

Maize leaves are organized into two major domains along the proximal-distal axis: a broad flat blade at the distal end of the leaf, and a narrow, thickened sheath that encircles the stem. Between the blade and sheath are two wedge-shaped tissues called auricles, and the ligule, an epidermally derived fringe. Members of the *Knotted1* (*Kn1*) family of mutations change the shape and position of both ligule and auricle, thus disturbing the overall pattern of the leaf. Here we present the results of a mosaic analysis of *Gnarley1-R* (*Gn1-R*), which like members of the *Kn1* family, affects the ligule and auricle. *Gn1-R* is distinct, however, in altering the dimensions of cells that make up sheath tissue. To gain insight into the *Gn1-R* phenotype, we performed a mosaic analysis using X-ray induced chromosome breakage to generate wild-type (*gn1+/-*) sectors in otherwise *Gn1-R* leaves. These sectors allowed us to determine whether *Gn1-R* acts non-autonomously to influence adjacent cells. Most aspects of the *Gn1-R* phenotype, such as ligule position, inhibition of auricle development, and sheath thickness showed autonomy in the lateral dimension (leaf width). In contrast, all aspects of the *Gn1-R* phenotype were non-autonomous in the transverse dimension (leaf thickness), suggesting that signaling occurs between cell layers in the leaf. These results support a model for distinct signaling pathways along lateral versus transverse axes of a developing leaf.

**Key words:** Maize, Leaf, Mosaic, *Gnarley1* (*Gn1*)

**INTRODUCTION**

How groups of initially undetermined cells develop into complex, patterned leaves is a central question in plant development. Each leaf first appears in a subapical position on the flank of the shoot apical meristem (SAM). This primordium becomes dorsoventrally flattened as it is displaced away from the shoot tip by additional leaf initiation events. By the time the leaf is the fifth from the meristem, it has distinct transverse axes of a developing leaf.

Clonal and histological analyses reveal patterns of cell divisions preferentially utilized during development. Newly initiated maize leaf primordia consist of a continuous epidermal layer derived from the L1 layer of the meristem and two internal layers derived from L2 cells (Sharman, 1942; Langdale et al., 1989). As primordia emerge from the SAM, one internal layer of cells, generally the adaxial layer, divides to give rise to the middle mesophyll layer from which the vascular elements differentiate (Langdale et al., 1989). Anatomical studies of maize leaves have shown that the midvein is the first vasculature to form in the leaf, followed by the lateral veins, both of which develop from leaf base to tip (Sharman, 1942; Esau, 1943). At the leaf tip, 5 to 12 intermediate veins initiate between the laterals and differentiate toward the leaf base. As these intermediates traverse the auricle region, they anastomose such that only one or two intermediate veins are found between lateral veins in the sheath.

Models to explain the types of signals involved in the coordinated development of the leaf have drawn heavily on the analysis of mutants (for review see Sylvester et al., 1996; Poethig, 1997). A number of dominant maize mutations have been characterized that specifically affect proximal-distal patterning (Freeling, 1992). *Knotted1* (*Kn1*), the first member of this family to be analyzed (Gelinas et al., 1969; Freeling and Hake, 1985), is characterized by sheath-like cells in the blade.
portion of the leaf (Sinha and Hake, 1994). Isolation of the gene showed that kn1 encodes a homeodomain protein which is normally expressed in the meristem, but excluded from leaves (Vollbrecht et al., 1991; Smith et al., 1992). The expression of kn1 in the blade of the dominant mutant leads to extra cell divisions in tissue surrounding lateral veins (Freeling, 1992). Ectopic expression of related knox genes (for kn1-like homeobox gene) in maize and tomato is also correlated with dominant leaf phenotypes characterized by failure to differentiate properly and by indeterminant patterns of growth (Schneeberger et al., 1995; Hareven et al., 1996; Chen et al., 1997; Parnis et al., 1997).

Further insight into the mechanisms by which specific genes act is provided by plants that are genetically mosaic. For example, the liguleless1 (lg1) and liguleless2 (lg2) mutations remove both the ligule and auricle. Sectors of lg1 mutant tissue in a lg1+ genetic background lack ligule and auricle, implying that the lg1 gene behaves cell autonomously (Becraft et al., 1990; Becraft and Freeling, 1991). In contrast, lg2 mutant sectors appear identical to adjacent lg2+ cells, thus the lg2 gene behaves non-autonomously (Harper and Freeling, 1996). These data suggest that LG1 may act as a receptor in the perception of a developmental signal involved with making ligule and auricle, whereas LG2 is more likely to participate in making or transporting the signal from cell to cell. Mosaic analysis is also informative in dissecting pleiotropic effects of a mutation and determining the foci for the mutant phenotype(s). For example, mosaic analysis of the lg1 mutation indicates that LG1 is required in the epidermis for normal ligule formation and in internal layers for auricle formation (Becraft et al., 1990; Becraft and Freeling, 1991).

Here we present the results of a mosaic analysis of the dominant Gnarlery1 (Gn1-R) mutation of maize. The altered pattern of leaf development of Gn1-R is reminiscent of the phenotypes that result from the ectopic expression of knox genes in which the position of ligule and auricle are altered. Gn1-R is unique in that it specifically alters the morphology of the entire sheath (Fig. 1B), apparently by changing parameters of cell growth (T. F. and S. H., unpublished data). We show that Gn1-R’s effect on sheath thickness, ligule position and auricle initiation is autonomous in the lateral dimension. In contrast, Gn1-R acts non-autonomously laterally (from midrib to margins) during auricle propagation and non-autonomously transversely (between cell layers) for all aspects of the mutant phenotype. These outcomes lend themselves to a growing picture of patterning in maize leaf development by implying that the domains of the leaf are specified by distinct signaling pathways in lateral and transverse dimensions.

MATERIALS AND METHODS

Genetic stocks and irradiation conditions

Gn1-R is a spontaneous dominant mutation recovered by Dr Tony Pryor (CSIRO, Canberra, Australia). An albino marker, w3, approximately 25 map units proximal to gn1+ on 2L, was used as a recessive marker for the mosaic analysis (Demerec, 1923; Lindstrom, 1924). Seeds segregating 1:1 for Gn1-R W3+/gn1+ w3 and gn1+ W3+/gn1+ w3 were imbibed for 72 hours, at 25°C, with constant agitation. Imbibed seeds were irradiated with 1,000-1,500 rads of hard x-rays at either Lawrence Berkeley Labs, Berkeley, CA, or at the Palmerston North Hospital, Palmerston North, New Zealand.

Sector analysis

Approximately 10,000 irradiated seeds were field grown in either Gill Tract, Albany CA, or in the field at the Institute of Developmental Phenomenology, Raumi, New Zealand. As plants were grown to maturity, they were examined periodically for white (w3−) sectors. Sected leaves were measured and examined for gross phenotype. Fully expanded leaves were hand sectioned and observed using a epifluorescence microscope with a G365 excitation filter, FT 395 dichroic beamsplitter, and a LP 420 barrier filter. Under these conditions, normal chloroplasts fluoresce bright red, and cell walls appear pale yellow. No chlorophyll autofluorescence is detected in w3− cells. The presence or absence of chlorophyll in epidermal layers was scored by inspecting guard cells, the only chloroplast-containing cell type in this tissue.

Microscopy

Hand-cut sections were made, stained in a 1% Acidine Orange solution for 5 minutes, then destained with several changes of water. Tissue was mounted in water on glass slides and observed with a Leica Scanning Confocal Microscope. Samples were exposed to an excitation energy of 568 nm, and a second filter separated emitted wavelengths into 2 channels, one collecting Acridine Orange emission, the second collecting chlorophyll autofluorescence. The two channels were superimposed to generate an image in which both cell walls and chlorophyll were clearly visible. Cell measurements were performed using data analysis software on a Silicon Graphics computer.

Leaf surfaces were examined by Scanning Electron Microscopy (SEM) using a modified replica technique (Sylvestre et al., 1990). Casts of epidermal surfaces were filled with high strength epoxy resin. These epoxy resin replicas were polymerized for 24-48 hours, then mounted on SEM stubs and sputter coated with 25 nm of gold in a Polaron E 5400 sputter coater. The specimens were observed using an ISI DS-130 SEM, operating at an accelerating voltage of 15 kV.

DNA blot analysis

DNA was collected from large gn1+ w3+/− sectors and adjoining gn1+ w3/Gn1-R W3+ tissue. Genomic DNA isolation and Southern blot analysis were performed as previously described (Greene et al., 1994). A 5′ subclone of the knox4 cDNA was used as a hybridization probe (Foster, 1997).

RESULTS

Sector genotypes

To determine whether Gn1-R affects adjacent cells in a non-autonomous manner and to examine in finer detail individual aspects of the Gn1-R phenotype, genetic mosaics of Gn1-R and gn1+ (wild-type allele) were generated. Tissue lacking the dominant Gn1-R allele was marked by w3, an albino mutation that maps approximately 25 map units proximal to Gn1 on 2L (Demerec, 1923; Lindstrom, 1924). Kernels of the following genotype, Gn1-R W3+/gn1+ w3, were x-irradiated to induce chromosome breaks. X-ray induced chromosome breaks proximal to W3+ result in albino clonal sectors which lack the dominant alleles Gn1-R and W3+, i.e., albino, wild-type (gn1+) sectors in a green, Gn1-R background (Fig. 1C).

A total of 137 hemizygous (gn1+/ w3−) sectors in 69 Gn1-R plants were obtained. Only sectors that extended at least the entire length of the leaf were examined. Sectors were compared to adjacent Gn1-R tissue for the following characteristics: (1) position of the ligule, (2) shape and position of the auricle, and (3) sheath expansion in the transverse and longitudinal
dimensions. Because all three components of the Gn1-R phenotype are somewhat variable in expressivity, only sectors located in a region expressing a given characteristic were scored with respect to that characteristic. Sector width ranged from 1 mm to 4.2 cm and had no apparent effect on whether sectors were scored as Gn1-R or wild-type for any of the phenotypes examined. Thirty-one sectors in 14 wild-type plants (gn1+ W3+/gn1+ w3) were examined as a control for the phenotypic effect of albinism and hemizygosity.

The large genetic distance between w3 and gn1+ (>25 map units) could permit interstitial deletions that would uncover w3 without removing Gn1-R. Deletion of the Gn1-R locus in albino sectors was confirmed by analyzing DNA from green and albino sectors for the presence of polymorphisms at knox4, which maps to Gn1-R (Foster, 1997). In all of the samples analyzed, DNA from green tissue contained two knox4 hybridizing bands, corresponding to gn1+ and Gn1-R alleles, while DNA from albino tissue had only one band, corresponding to the hemizygous gn1+ allele (data not shown).

**Fig. 1.** Clonal analysis of Gn1-R. (A) The transverse, lateral and proximal-distal axes of the maize leaf. (B) Gnarley1 sheaths. On the left is the sheath portion of a wild-type leaf. On the right is a sheath of a Gn1-R plant. The Gn1-R sheaths are shorter and wider than normal. (C) Schematic diagram of clonal analysis. The X-ray-induced chromosome breakage depicted results in the loss of the dominant alleles Gn1-R and W3+, and reveals the recessive gn1+ and w3 phenotypes. Clones derived from such an event appear as non-gnarley, i.e., wild-type (gn1+), white sectors in gnarley (Gn1-R), green leaves. Other chromosome breaks that randomly occur do not produce white sectors.

**Ligule position**

The ligule is an epidermally derived fringe that forms at the boundary between sheath and blade. Ligules normally develop in a horizontal line across the leaf width. In Gn1-R mutants, the ligule does not form in a straight horizontal line, but instead is always positioned more distally on tissue immediately flanking the midrib than over the midrib itself (Foster, 1997).

Of 121 scorable sectors, 73% (88/121) displayed a ligule position that was more proximal relative to the ligule position in adjacent Gn1-R tissue. 24% of the sectors (29/121) showed a ligule position similar to that in adjacent Gn1-R tissue and 3% (4/121) displayed distal repositioning of ligule relative to adjacent Gn1-R tissue (Table 1). The fact that the majority of experimental sectors showed a more proximally positioned ligule suggests that the Gn1-R mutation acts to displace ligule distally. We classified the sectors that had a more proximal ligule as gn1+, and the other sector types as phenotypically Gn1-R (Fig. 2). The abrupt repositioning of the ligule in a majority of sectors suggests that Gn1-R acts autonomously in the lateral direction with respect to ligule position.

In most cases, the ligule ran along one or both sector borders (Fig. 3A and B). These sector borders were generally located at a lateral or large intermediate vein. In 15 sectors, the proximal repositioning of the ligule occurred within the sector, rather than precisely at the sector boundary. The sector borders for 12 of these 15 sectors, whose composition included both completely albino and mixed mesophyll types, were located between a lateral and large intermediate vein. Thus, sector borders located at major veins tended to reveal a sharp change in ligule position, whereas sector borders located between major veins showed a gradual change in ligule position.

In both normal and Gn1-R leaves, cells distal to the ligule differentiate into blade, while cells proximal to the ligule become sheath (Sharman, 1942; Sylvester et al., 1990). Blade and sheath are composed of tissues with distinct epidermal and internal characteristics (Sylvester et al., 1990; Sinha and Hake, 1994). As evidenced by light microscopy and SEM analysis of epidermal characters, gn1+ w3/– sectors that moved the ligule proximally displayed concomitant changes in both histological organization and cell differentiation. Fig. 3C-D illustrates ligule positioned parallel to the sector boundary, accompanied by a lateral transition from albino gn1+ w3 blade to green Gn1-R W3+ sheath. Thus, the distinction between blade and sheath is sharply demarcated regardless of the boundary shape and, the ligule position adheres to this boundary.

Sectors with one or two layers of Gn1-R W3+ mesophyll tissue often flanked completely albino tissue. These sectors allowed us to determine whether there was a difference between the frequency of proximal ligule repositioning in albino mesophyll versus mixed albino/green mesophyll sectors. Very little difference was found between these sector types, and similarly, there was no difference in the frequency of ligule repositioning in sectors with a Gn1-R epidermis versus those with a gn1+ epidermis (Table 1 and Fig. 2). The position of the ligule in mixed mesophyll sectors was the same as in completely albino sectors, suggesting that the position of the ligule and specification of cell identity within internal tissue is influenced by non cell-autonomous signaling between layers of the leaf (Table 2).
Auricle position and shape

The normal maize auricle extends from either side of the midrib to the leaf margins in a triangular-shaped wedge, immediately distal to the ligule (Fig. 4D). It is thought that the auricle initiates at the midrib and propagates toward the margin (Becraft and Freeling, 1991). Auricles in Gn1-R leaves are generally reduced to smaller, irregularly shaped wedges that are displaced towards the margins or are eliminated altogether in severe Gn1-R mutants (T. F., B. V. and S. H., unpublished data).

Of 135 sectors, 22 contained a normal-appearing auricle in which the narrow point of the auricle was located at the midrib side of the sector boundary as shown in Fig. 4A,B. The sharp transition between green Gn1-R W3+ sheath and albino gn1+ w3 auricle was apparent in cross section; sheath tissue has large mesophyll cells and a hairless adaxial epidermis, whereas auricle cells are thick-walled and long hairs develop from both the adaxial and abaxial epidermis (Fig. 4C). The initiation of auricle at the sector boundary was always associated with proximal ligule repositioning at the same border (Fig. 4B).

In 10 of the 22 sectors that were surrounded by Gn1-R tissue on both the midrib and margin sides, normal-appearing auricle continued into mutant tissue on the margin side (Fig. 4E-F). In these 10 sectors, the ligule was displaced proximally at the midrib side of the sector border and continued into mutant tissue in a position immediately proximal to the auricle. The auricle continued into mutant tissue in a smooth line; it did not reinitiate or change shape at the Gn1-R border. This finding provides strong support to the idea that the auricle initiates near the midrib and propagates toward the margin (Becraft and Freeling, 1991). This result also suggests that the two aspects, initiation and propagation of auricle, are distinct. The auricle requires wild-type cells to initiate, but can propagate into Gn1-R cells.

The only sectors able to correctly initiate auricle were albino in all internal layers (Table 1 and Fig. 2). The fact that we did not find any auricle initiation within mixed green/albino mesophyll sectors suggests that Gn1-R can act in any internal layer to retard auricle initiation. We conclude that some component of the mechanism by which Gn1-R inhibits auricle initiation is autonomous in the lateral dimension, but non-autonomous in transverse dimension. In addition, once the auricle is initiated in a wild-type sector, it can be propagated into mutant tissue. Thus wild-type tissue acts non-autonomously in the lateral dimension to propagate the auricle (Table 2).

Sheath thickness and length

Vascular bundles in the sheath of normal maize leaves are appressed against the abaxial epidermis and are associated with hypodermal sclerenchyma that is continuous with the abaxial surface (Russell and Evert, 1985). Gn1-R mutants exhibit an irregular organization of sheath tissue; veins are located at a more median position between adaxial and abaxial surfaces, and the associated hypodermal sclerenchyma is not continuous between vascular bundles and the abaxial surface (Foster, 1997). Proliferation of enlarged mesophyll cells, particularly in the abaxial layer, result in thickened sheath tissue and abnormal histology in Gn1-R sheaths (Fig. 5A and B).

Of the 112 sectors with an entirely albino (gn1+ w3) mesophyll, 91% (102/112) had sheath tissue that was thinner in cross section than adjacent Gn1-R tissue, whereas 8% (9/112) were the same abnormal thickness as adjacent Gn1-R tissue (Table 1). Sectors exhibiting a reduction in sheath thickness were easily detected macroscopically, and further examined in cross section (Fig. 5B,C). Analysis by scanning confocal microscopy confirmed that abaxial mesophyll cells within thin sheath sectors were similar in size to comparable wild-type abaxial mesophyll cells (data not shown). Thus, sectors with reduced sheath thickness were recorded as wild type (gn1+) for this aspect of the Gn1-R phenotype and sectors showing overexpansion of mesophyll cells were recorded as Gn1-R. Our data indicate that the excessive growth of sheath mesophyll conditioned by the Gn1-R mutation is confined to cells carrying the Gn1-R mutation and does not affect laterally adjacent wild-type cells.

Sectors containing one or more internal layers of mutant tissue produced mixed results, but in general, the tissue thickness was the same as adjacent mutant tissue. No single layer appeared responsible for causing the mesophyll overgrowth phenotype (data not shown). This trend suggests
that *Gn1-R* may act non-autonomously in the transverse dimension to cause the cell shape defect in the sheath (Table 2).

Cell measurement data indicates that *Gn1-R* sheath cells are shorter in the proximal-distal dimension than comparable wild-type sheath cells (T. E., B. V. and S. H., unpublished data). If the *Gn1-R* cell elongation defect was confined to cells carrying the mutation, one might expect to see some differences in cell elongation between wild-type sectors and adjacent *Gn1-R* tissue. No evidence of shearing or buckling at the sector boundaries was observed in any of the 137 experimental sectors observed, including 32 marginal sectors. These data imply that the sheath cell elongation defect of *Gn1-R* is non-autonomous, i.e., *gn1*+ sheath cells fail to elongate correctly in *Gn1-R* leaves.

In order to control for the possibility that hypoploidy for chromosome 2L was responsible for the differences observed between *gn1*+ *w3/+* sectors and adjacent *Gn1-R/gn1*+ tissue, 31 albino sectors in control plants (*gn1*+ *W3+/gn1*+ *w3*) were also examined. Neither the auricle nor ligule were affected by any of these control sectors (data not shown) nor did any of the control sectors display a reduction in sheath thickness (Fig. 5D), ruling out the possibility that the changes observed in experimental sectors were caused by hypoploidy for 2L within the sector.

### DISCUSSION

We undertook a mosaic analysis of *Gn1-R* leaves in order to dissect out separate aspects of the pleiotropic phenotype and to determine the extent to which they are determined in a cell autonomous manner. The pleiotropic effects of the *Gn1-R* mutation on maize leaf development can be interpreted as two distinct defects; a pattern defect which affects the shape and position of the ligule and auricle, and a cell dimension defect which specifically affects sheath tissue. These two defects do not appear to be causally related, as evidenced by the behavior of wild-type sectors within *Gn1-R* leaves. The data reveal that ligule positioning, auricle formation and sheath shape can be uncoupled and that each of these tissues are uniquely specified by signals in the lateral and transverse dimension.

### Lateral veins function as compartment boundaries

The ligule and associated blade/sheath boundary in 73% of the

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**Table 1. Tissue layer compositions of Gn1-R sectors and their phenotypic effects**

<table>
<thead>
<tr>
<th>Sector composition*</th>
<th>n</th>
<th>Ligule positioning‡</th>
<th></th>
<th>Auricle initiation§</th>
<th></th>
<th>Sheath thickness¶</th>
</tr>
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<tbody>
<tr>
<td>W-W-W-W-W</td>
<td>44</td>
<td>13</td>
<td>27</td>
<td>4</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>G-W-W-W-G</td>
<td>68</td>
<td>15</td>
<td>43</td>
<td>10</td>
<td>58</td>
<td>10</td>
</tr>
<tr>
<td>W-G-W-W-G</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>G-G-W-G-G</td>
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<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>G-W-W-G-W</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G-W-G-G-G</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>W-G-W-G-G</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>G-W-W-G-G</td>
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<tr>
<td>W-G-G-W-W</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>G-G-G-G-G</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

*From left to right, the layers are abaxial epidermis, abaxial mesophyll, middle mesophyll, adaxial mesophyll, adaxial epidermis (Langdale et al., 1989). W signifies the loss of *Gn1-R W3+,* uncovering *gn1*+ *w3*. G signifies the presence of *Gn1-R W3+.*

‡Ligule position was scored as *Gn1-R* if the ligule in *gn1*+ *w3* sectors was positioned distally relative to ligule in adjacent *Gn1-R* tissue or was the same as adjacent tissue. Ligule position was scored as + if the ligule in *gn1*+ *w3* sectors was positioned proximally relative to ligule in adjacent *Gn1-R W3+* tissue. Due to ligule position irregularities near the midrib of *Gn1-R* mutants, *gn1*+ *w3* sectors within 5 mm of the midrib were not scorable for this phenotype (N/S).

§Auricle initiation was scored as *Gn1-R* if a wedge-shaped auricle did not initiate within the *gn1*+ *w3* sector. Auricle initiation was scored as + if a wedge-shaped auricle initiated at the midrib side of the *gn1*+ *w3* sector boundary. If an auricle had been initiated in *Gn1-R W3+* tissue on the midrib side of the *gn1*+ *w3* sector, the auricle initiation phenotype was not scorable (N/S).

¶Sheath thickness was scored as *Gn1-R* if the *gn1*+ *w3* sheath tissue was abnormally thick in the transverse dimension as adjacent *Gn1-R W3+* tissue. Sheath thickness was scored as + if *gn1*+ *w3* sheath tissue was less thick in the transverse dimension than adjacent *Gn1-R W3+* tissue. *gn1*+ *w3* sectors located in a region where the mesophyll overgrowth phenotype was not expressed could not be scored for this phenotype (N/S).

**Table 2. Summary of mosaic analysis of Gn1-R**

<table>
<thead>
<tr>
<th>Character</th>
<th>Lateral dimension</th>
<th>Transverse dimension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligule position</td>
<td>Autonomous</td>
<td>Non-autonomous (wild-type)</td>
</tr>
<tr>
<td>Initiation of auricle</td>
<td>Autonomous</td>
<td>Non-autonomous (<em>Gn1-R</em>)</td>
</tr>
<tr>
<td>Propagation of auricle</td>
<td>Non-autonomous (wild-type)</td>
<td>Non-autonomous (<em>Gn1-R</em>)</td>
</tr>
<tr>
<td>Sheath thickness</td>
<td>Autonomous</td>
<td>Non-autonomous (<em>Gn1-R</em>)</td>
</tr>
</tbody>
</table>

The effect of *Gn1-R* on a given character was considered autonomous if the phenotypes at a sector border were determined by their respective genotypes and non-autonomous if the phenotype was influenced by adjacent cells. The lateral dimension is midrib to margin and the transverse dimension is adaxial epidermis to abaxial epidermis. Depending on the character and dimension being analyzed, the *Gn1-R* tissue influenced adjacent wild-type cells and wild-type tissues influenced adjacent *Gn1-R* cells. The genotype that exerts the influence is indicated in parentheses.
at the boundary between sheath and \textit{Gn1-R} ligule; \textit{s}, sheath. Scale bar, 200 μm.

**Fig. 3.** Proximal ligule position in a \textit{gn1+/w3l−} sector. Abaxial (A), and adaxial (B) views of a leaf with a sector exhibiting proximal ligule repositioning coincident with sector borders. (C) Cross section through the sector boundary indicated by the box in B, with the adaxial surface oriented towards the top of the page. Under UV fluorescence, green (\textit{Gn1-R W3+}) cells appear red due to autofluorescence, and albino (\textit{gn1+/w3}) cells appear green. Note the sharp boundary between \textit{Gn1-R W3+} cells that exhibit sheath histology and \textit{gn1+/w3} cells that exhibit blade histology. (D) SEM of the adaxial epidermis of the area indicated by the box in B. \textit{Gn1-R} tissue has the smooth, rectangular cells characteristic of sheath, while the \textit{gn1+} sector has epidermal features of blade tissue. Ligule forms at the boundary between \textit{Gn1-R} sheath and \textit{gn1+} blade. \textit{b}, blade; \textit{lg}, ligule; \textit{s}, sheath. Scale bar, 200 μm.

Wild-type (\textit{gn1+}) sectors was moved to a more proximal position relative to the ligule in adjacent \textit{Gn1-R} tissue. This finding suggests that \textit{Gn1-R} displaces the ligule in a distal direction, similar to the effect that other dominant \textit{Knox} mutations have on the ligule position (Freeling, 1992). In most cases, the ligule position was coincident with sector boundaries, indicating that factors causing the distal displacement of \textit{Gn1-R} ligule must act autonomously in the lateral direction. In a small number of sectors, however, the ligule was not coincident with the sector boundary, but was positioned at an angle to the sector border. The majority of these sectors had boundaries located between lateral veins.

A possible explanation for the difference in autonomy dependent on position of sector boundaries comes from clonal analysis which suggests that the tissue between lateral veins represents a developmental compartment (Cerioli et al., 1994). Analysis of the epidermis indicates that the \textit{Gn1-R} ligule is frequently disrupted at lateral veins, but is continuous between lateral veins. In light of this observation, \textit{Gn1-R} might act to reposition the location of the blade-sheath boundary within units of lateral veins. Wild-type sectors with borders that are coincident with a lateral vein may be able to correctly interpret developmental signal(s) and initiate ligule at a more appropriate (proximal) location precisely at the sector boundary. Sectors with borders positioned between lateral veins may have a more gradual displacement of ligule due to diffusion of signals between lateral veins in mutant and wild-type tissue. Clonal analysis of the maize \textit{Liguleless3 (Lg3)} mutation supports such a model (Fowler et al., 1996).

**One class of sectors reveals a distinction between signal initiation and propagation**

In addition to the ligule position defect, \textit{Gn1-R} suppresses the initiation of auricle near the midrib, and interferes with the longitudinal expansion of the auricle as a whole. This aspect of the \textit{Gn1-R} phenotype is variable in expression and is correlated with severity of the overall mutant phenotype (Foster, 1997). In highly expressive leaves, the auricle is reduced to small, irregularly shaped patches near the leaf margin or is absent altogether. In a small, but significant fraction of \textit{gn1+} sectors, a normal shaped auricle initiated exactly at the midrib side of the sector boundary and only at this position. These auricles were associated with proximal ligule repositioning along that sector border. These results demonstrate that inhibition of auricle initiation by \textit{Gn1-R} is autonomous in the lateral dimension. However, the large fraction of sectors in which no auricle was initiated suggests that additional factors may influence auricle initiation that were not revealed by the sector analysis.

The observation that auricle initiated at the \textit{gn1+} sector boundary continued into \textit{Gn1-R} tissue on the margin side suggests that initiation and propagation of auricle can be separated. Either auricle propagation is autocatalytic once initiated, or there are two separate signals, one that initiates auricle, and a second that propagates it towards the margins. These data suggest that \textit{Gn1-R} retards the initiation, but not the continuation of auricle. Thus, the propagation of a ‘continue-auricle’ signal from the midrib towards the margins is independent of the presence of the \textit{Gn1-R} genotype. The results also underline a difference between positioning the ligule that forms in the absence or presence of an auricle. Ligules without auricles, that were repositioned to a more proximal position in a sector, returned to the \textit{Gn1-R} position outside the sector, in contrast, ligules that initiated with auricles in a sector, continued with the auricle in the \textit{gn1+} position into \textit{Gn1-R} tissue.

Sectors that influenced auricle initiation were also described by Becraft and coworkers in their mosaic analysis of the \textit{lg1} mutation (Becraft and Freeling, 1991). In these studies, ligule and auricle were interrupted by sectors of \textit{lg1−} mutant tissue in an otherwise normal leaf. In wild-type tissue on the marginal side of the \textit{lg1−} sector, both ligule and auricle were reinitiated and repositioned in a more proximal position relative to ligule and auricle on the midrib side of the sector. The proximal repositioning occurred in 40% of the sectors and then only when all layers were \textit{lg1−}. The distance by which the ligule was repositioned did not correlate with sector width nor with position relative to the midrib, leading the authors to interpret the repositioning as a reinitiation of ligule and auricle, rather than a continuation of auricle from the midrib side of the sector (Becraft and Freeling, 1991). This distinction is important because it implies that, in addition to a requirement for making ligule and auricle, LG1 plays a role in the propagation of a
‘make ligule-make auricle’ signal, a signal that must travel from the midrib towards the margins.

It is of interest that the position of wild-type ligule is more proximal whether it is conditioned by the hemizygous gn1+ sector in a Gn1-R leaf or conditioned by the Lg1+/lg1 tissue adjacent to the hemizygous lg1 sector. This finding could reflect the differentiation of leaf cells that follows two gradients of maturation, one from the tip to the base, and one from the midrib to the margins (Sylvester et al., 1990; Hagemann and Gleissberg, 1996). Cells that are delayed in maturation in the proximal-distal gradient may still receive signals propagated in a lateral direction of maturation and vice versa, thus explaining displacement of pattern boundaries within the leaf.

Both autonomous and non-autonomous factors cause abnormal sheath shape in Gn1-R mutants

Gn1-R sheath tissue is abnormally proportioned in length, width and thickness. Shortened sheaths are correlated with an overall reduction in sheath cell lengths in the proximal-distal dimension (T. F., B. V. and S. H., unpublished data). The cell elongation defect appears to act entirely non-autonomously, as cells in wild-type sectors did not overgrow mutant tissue, even in marginal sectors where tissue is only physically constrained on one side. A mosaic analysis of the dominant dwarf mutant, D8, yielded similar results. Like many of the maize dwarfs, D8 plants have smaller cells than normal (Harberd and Freeling, 1989; Freeling, 1992). Sectors of wild-type tissue in D8 leaves rarely grew more than adjacent mutant tissue. Harberd and Freeling suggest a ‘default cell division rule’, which would function to keep slower or faster growing cells in check with the majority of cells in order to facilitate the coordinated growth of the organ. The results of our mosaic analysis of Gn1-R can be explained by such a model. In contrast to these results with maize leaves, clonal analysis of the cotton Okra mutant showed striking differences in growth between lobes of leaves, suggesting that leaf shape...
coordination could be uncoupled in this species (Dolan and Poethig, 1998).

In contrast to the lack of autonomy seen in the sheath length, the effect Gnl-R has on sheath thickness was autonomous in the lateral dimension. The great majority of sectors were reduced in sheath thickness beginning at the sector boundary. In cross section, these wild-type sectors did not exhibit the abnormally large mesophyll cells seen in adjacent mutant tissue. Thus, the cell shape defect is laterally autonomous when growth is not constrained by adjacent tissue.

Periclinal sectors reveal signaling between layers
A number of sectors were composed of both green and albino layers (Table 1). These sectors, referred to as periclinal sectors, help us to determine the effect the dominant Gnl-R mutation has on adjacent gn1+ layers and vice versa. One class of periclinal sectors analyzed contained a Gnl-R epidermis over wild-type internal tissue. With respect to all aspects of the phenotype, including the position of the ligule, which is an epidermally derived tissue, the Gnl-R epidermis had no effect. These data indicate that internal layers of wild-type tissue may act non-autonomously to elicit changes in a Gnl-R epidermis. Mosaic analyses of Lg3 in maize and fasciated in tomato provide a precedent for internal layers influencing the fate of epidermal layers (Fowler et al., 1996; Szymkowiak and Sussex, 1992). However, mosaic analysis of the cotton leaf mutant, Okra, demonstrates a significant influence of mutant epidermis on wild-type internal layers (Dolan and Poethig, 1998), suggesting that the influence of the epidermal genotype depends on the particular gene. One factor that could be significant in this distinction is the site of gene expression. For example, the expression of knl in Knl-N leaves is confined to the vascular cells, although protein has been localized to the epidermis (Smith et al., 1992; Jackson et al., 1994). It has been suggested that the presence of the protein in the epidermis is due to trafficking of the protein (Lucas et al., 1995). Given this information, we would not expect to see a mutant phenotype if the epidermis is the only layer to carry the Knl-N allele (Hake and Freeling, 1986; Sinha and Hake, 1990), similarly, if Gnl-R does not condition ectopic RNA expression in the epidermis, we would not expect the epidermis of the genotype to be a determining factor.

Another class of periclinal sectors contained both Gnl-R and gn1+ mesophyll layers. We discovered that both Gnl-R and gn1+ layers influenced adjacent layers, depending on the particular aspect of the phenotype under observation. The presence of a Gnl-R layer in a mixed mesophyll sector was sufficient to prevent auricle initiation and tended to result in enlarged sheath cells. Thus, Gnl-R acts non-autonomously in regards to these two aspects of the phenotype. The non-autonomy could be due to the movement to adjacent cell layers of KNOX proteins or gene products under their transcriptional control. The wild-type (gn1+) layer correctly positioned the ligule, despite adjacent Gnl-R layers. This result suggests that wild-type leaf cells produce a diffusible product that establishes the position of the ligule regardless of the presence of Gnl-R in adjacent layers.

Mosaic analyses of Gnl-R and Rs1 differ in autonomy of mutant phenotypes
Certain aspects of the Gnl-R phenotype, such as the distal repositioning of ligule, transverse thickening of sheath tissue, and disruption to auricle development bear a striking resemblance to the phenotype conditioned by the Rs1 mutation (Becraft and Freeling, 1994). Gnl1 and Rs1 map to chromosomal regions that share extensive synteny (2L and 7S, respectively) and are likely to be the result of an ancient duplication (Kerstetter et al., 1994). knox4 is tightly linked to Gnl-R (Foster, 1997) and encodes a polypeptide with 81% identity to RS1 (Schneeberger et al., 1995; J. Yamaguchi and S. H., unpublished data). Thus, knox4 and rs1 are likely to be duplicate genes.

A mosaic analysis of the Rs1 mutation revealed that all aspects of the mutation were non-autonomous in both lateral and transverse dimensions (Becraft and Freeling, 1994). This result suggests that RS1 itself or a downstream molecule moves between cells and induces genetically wild-type tissue to develop Rs1 mutant characteristics. Given the similarities between Gnl-R and Rs1 mutant phenotypes, the results of this mosaic analysis of Gnl-R are somewhat surprising. Mutant phenotypes that behave in a cell autonomous manner are generally indicative of gene products that function in perception of developmental signals, rather than genes that function in long distance signaling. This finding suggests that Gnl-R acts to interfere with the perception of developmental signals. One possible explanation for the difference in autonomy could be the fact that ectopic expression of rs1 is detected in veins (Schneeberger et al., 1995), whereas, ectopic expression of KNOX protein is not detected in veins of Gnl-R leaves (T. F., B. V. and S. H., unpublished data). Perhaps the vein expression triggers some transmissible signal.

Insights into normal maize leaf development
Our analysis of wild-type sectors within Gnl-R leaves reveals some of the interplay that occurs in establishing the normal maize leaf. A role for lateral veins in establishing position of the ligule is suggested by the Gnl-R sector boundaries; the ligule followed the sector border when the sector occurred at a vein, but not when the sector fell between veins. We would also predict that the ligule position is determined by signaling that occurs within layers of the leaf, in that wild-type layers influence adjacent Gnl-R layers to establish position of the ligule. The initiation of the auricle, on the other hand, was inhibited by the presence of Gnl-R layers. This uncoupling of ligule and auricle only occurs when the ligule exists in the absence of auricle. Our data support the idea, predicted by Becraft and Freeling (1991), that the auricle initiates at the midrib and differentiates toward the margin. We have added to that concept by showing that propagation of the auricle is separable from initiation of the auricle.

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Mosaic analysis of Gnarley1 313


