Characterisation of the maize leaf patterning mutants
*Wavy auricle in blade1-R* and *milkweed pod1-R*

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Abstract

The maize leaf has three main axes of growth, with an asymmetric distribution of tissue types along each axis. This study focuses on three mutants, Wavy auricle in blade1-R (Wab1-R), liguleless1-R (lg1-R) and milkweed pod1-R (mwp1-R) that disrupt axial patterning of maize leaves. Dominant Wab1 mutations disrupt both medial-lateral and proximal-distal patterning. Wab1 leaf blades are narrow and ectopic auricle and sheath-like tissues extend into the leaf blade. Previous analyses have shown that Lg1 acts cell-autonomously to specify ligule and auricle tissues. The current study reveals additional roles in defining leaf shape. The recessive lg1-R mutation exacerbates the Wab1-R phenotype; in the double mutants, most of the proximal blade is deleted and sheath tissue extends along the residual blade.

A mosaic analysis of Wab1-R was conducted in Lg1 and lg1-R backgrounds to determine if Wab1-R affects leaf development in a cell-autonomous manner. Normal tissue identity was restored in all wab1/- sectors in a lg1-R mutant background, and in three quarters of sectors in a Lg1 background. These results suggest that Lg1 can influence the autonomy of Wab1-R. In both genotypes, leaf-halves with wab1/- sectors were significantly wider than non-sectored leaf-halves, suggesting that Wab1-R acts cell-autonomously to affect lateral growth.

mwp1-R is a recessive mutation that specifically affects patterning of sheath tissue. Characterisation of the mwp1-R phenotype revealed that mwp1-R husk leaves and the sheaths of vegetative leaves develop pairs of outgrowths on the abaxial surface associated with regions of adaxialised tissue. In situ hybridisation confirmed that disruptions to adaxial-abaxial patterning are correlated with misexpression of leaf polarity genes. Leaf margins and fused organs such as the prophyll are most severely affected by mwp1-R. The first two husk leaves normally fuse along adjacent margins to form the bi-keeled prophyll. In the most severe cases the mwp1-R prophyll is reduced to an unfused, two-pronged structure and keel outgrowth is significantly reduced. We speculate that the adaxial-abaxial patterning system has been co-opted during evolution to promote outgrowth of the keels in normal prophyll development.
The results of this study place $Mwp1$, $wab1$ and $Lg1$ in a network of genes that regulate leaf polarity and axial patterning.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
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<td>dicotyledon</td>
</tr>
<tr>
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<td>digoxigenin</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPX</td>
<td>dibutylphthalate polystyrene xylene</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FAA</td>
<td>formaldehyde, acetic acid, ethanol</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>KV</td>
<td>kilovolt</td>
</tr>
<tr>
<td>L1, L2, L3</td>
<td>cell layers in the shoot apical meristem and lateral organs</td>
</tr>
<tr>
<td>LM</td>
<td>lateral meristem</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>miRNA</td>
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<td>monocotyledon</td>
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<td>messenger ribonucleic acid</td>
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<tr>
<td>NBT/BCIP</td>
<td>5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride</td>
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<td>nanometre</td>
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<tr>
<td>NTP</td>
<td>nucleotide triphosphates</td>
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<tr>
<td>P0, P1, P2</td>
<td>plastochron number</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SAM</td>
<td>shoot apical meristem</td>
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SEM  scanning electron microscopy
SSC  sodium chloride, sodium citrate
SSPE sodium chloride, sodium phosphate, EDTA
TBS  tris buffered saline
TE   tris, EDTA
tRNA transfer ribonucleic acid
µg   microgram
µl   microlitre
µm   micron
w/v  weight by volume ratio
v/v  volume to volume ratio
1. Introduction

1.1 Background

A central issue in developmental biology is how pattern is superimposed on initially uniform groups of cells (Russo, 1999; Gilbert, 2000). A primary mechanism in animal development is the subdivision of fields of cells into smaller compartments defined by differential gene expression (Lawrence and Struhl, 1996). New axes of growth can form where two compartments are juxtaposed (Diaz-Benjumea and Cohen, 1993).

There is evidence that similar mechanisms operate in leaf development. Distinct cell types develop on the adaxial (upper) and abaxial (lower) surfaces of most leaves (Steeves and Sussex, 1984). The complete loss of either domain results in radial leaves whereas partial loss generates new boundaries, resulting in ectopic outgrowths (Sussex, 1954; Waites and Hudson, 1995; McConnell and Barton, 1998; Eshed et al., 2001). Many genes involved in the establishment of leaf polarity have been identified in dicots (McConnell and Barton, 1998; Alvarez and Smyth, 1999; Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; McConnell et al., 2001). However, mechanisms controlling leaf polarity in monocots are less well known.

One approach that has been successful in elucidating the mechanisms that control development is the study of mutants that exhibit developmental defects. The current study focuses on two maize mutants that disrupt leaf polarity. Wavy auricle in blade1-R (Wab1-R) is a dominant mutation that disrupts medial-lateral and proximal-distal patterning of leaves, resulting in narrow leaves and inappropriate cell differentiation (Hay and Hake, 2004). I have used mosaic analysis to investigate the mode of action of Wab1-R and to elucidate genetic interactions between Wab1-R and Liguleless1 (Lg1).

The recessive milkweed pod1-R (mwp1-R) mutation was first identified by ectopic outgrowths on the abaxial surface of the husk leaves. This phenotype suggested that Mwp1 may be required for the establishment or maintenance of
leaf polarity. The \textit{mwp1-R} mutation specifically affects sheath tissue, the basal part of the lower leaf zone. This part of the leaf is extremely reduced in dicots (Troll, 1955; Kaplan, 1973). The \textit{mwp1-R} phenotype is particularly severe in the prophyll and silks – organs that are thought to form via phytomer fusion (Bossinger \textit{et al.}, 1992). Thus, this mutant affords the opportunity to investigate the establishment of polarity in lateral organs with diverse morphologies.

1.2 Growth of the maize plant

In maize, the primary axis is generated by a single shoot apical meristem (SAM). The SAM initiates a series of vegetative leaves in an alternate pattern before terminating in the tassel (male inflorescence) (Poethig, 1994).

The male and female inflorescences are borne separately. The female inflorescences (ears) arise from meristems in the axils of vegetative leaves at the upper nodes. The SAM terminates in the tassel after the production of vegetative leaves has ceased. Flowers are initially bisexual and become unisexual by the abortion of gynoecia in male flowers, and of stamens in female flowers.

The early stages of spikelet and flower development are similar for male and female flowers (Cheng \textit{et al.}, 1983). Inflorescence meristems produce spikelet pair primordia. Each spikelet pair primordium produces two spikelet primordia which in turn initiate two florets each. There are two flowers per male spikelet. In female spikelets, the lower floret is repressed so that only one functional floret develops per spikelet. The spikelet primordia each initiate a series of bract-like organs - two glumes and two lemmas - before the lower floret is initiated in the axil of the outer lemma. Each floret meristem initiates a bi-keeled palea then lodicules, stamens and the gynoecium. The gynoecium is formed by the outgrowth of a ridge which overgrows the apical meristem, giving rise to the stylar canal and silk. In maize, the styles are fused and very short. The stigmas are very long and can reach up to 75cm. They arise close to the ovary and are fused for most of their length, bifurcating at the very tip (Clifford, 1988). There is evidence that the silks are formed via the preprimordial fusion of the two
posterior carpels along two sets of margins (see Figure 1.6 D) (Cronquist, 1988; Scanlon and Freeling, 1998).

1.3 Leaves have three main axes of growth

Leaves have three main axes of growth defined with reference to the SAM. These are the proximal-distal axis, the adaxial-abaxial axis and the medial-lateral axis (Figure 1.1). The proximal end is attached and closest to the SAM, while the distal end is unattached. The adaxial surface of a leaf is the one adjacent to the SAM, generally the top surface of the mature leaf, while the abaxial surface is the one furthest from the SAM. The medial-lateral axis is defined as midrib to margin. In most species there are characteristic anatomical and morphological asymmetries along each axis that specialise different regions of the leaf for particular functions (Sylvestre et al., 1990; Kerstetter et al., 2001).

Figure 1.1. Leaf axes. Axes of growth of dicot (left) and monocot (right) leaves. AD-AB = adaxial-abaxial M-L = medial-lateral.

The maize leaf is divided into sheath and blade along the proximal-distal axis (Figure 1.1). The sheath wraps tightly around the culm (stem) of the plant and is separated from the blade by a fringe of ligule tissue and two wedges of auricle tissue. Each of the tissue types has characteristic epidermal and anatomical features (Sylvestre et al., 1990). The adaxial blade surface is characterised by rows of bulliform cells and three types of hair. Cell wall junctions are crenulated and interlocking. Sheath cells are non-crenulated and elongated, the abaxial surface has long hairs whereas the adaxial surface has shorter prickly-type hairs. The leaves of dicots such as Arabidopsis have a proximal petiole and a
distal lamina. Dicot petioles are often radial in transverse section, whereas the blade has distinct adaxial and abaxial surfaces (Kerstetter et al., 2001).

1.4 Leaf initiation and early development

1.4.1 Founder cells

Morphological differences in the development of dicot and grass leaves are apparent from the time the leaf primordia emerge. Dicot leaf primordia emerge as peg-like outgrowths on the flanks of the SAM, whereas the maize leaf primordium emerges as a ridge of cells that encircles the SAM. Differences in the morphology of dicot and grass leaf primordia reflect differences in the distribution of leaf founder cells (Figure 1.2). The term "founder cell population" refers to those cells within the meristem that are specified to become incorporated into a lateral organ (Poethig, 1984).

In Arabidopsis, a small group of about 30 leaf founder cells is recruited, while in tobacco, the founder cell population is about 50-100 cells (Poethig and Sussex, 1985a; Irish and Sussex, 1992). In maize, the founder cell number is estimated to be about 200 cells (Poethig and Szymkowiak, 1995). Leaf founder cells in dicots occupy only a fraction of the circumference of the SAM, whereas in maize, founder cells are recruited from the entire circumference of the SAM (Figure 1.2). Initialisation of leaf founder cells in maize begins at the future midrib, and is propagated outward toward the margins (Sharman, 1942). The domains that give rise to the sheath margins overlap, so that clonal sectors arising in this part of the SAM include both
margins of the sheath (Poethig, 1984). A number of maize mutants have narrow leaves due to defects in founder cell recruitment (Timmermans et al., 1998; Scanlon, 2000).

Clonal analysis has been used to trace cell lineage and patterns of cell division, both within the meristem and in developing organs, and to estimate leaf founder cell numbers (Steffensen, 1968; Poethig and Sussex, 1985b; Irish and Sussex, 1992; Poethig and Szymkowiak, 1995). Clonal analysis requires that a cell be marked with a heritable, visible, cell-autonomous marker. This allows derivatives of the marked cell to be identified (Figure 1.3). Hyperploidy has been used as a marker of cell lineage within the SAM (Steeves and Sussex, 1984). Albino mutations are commonly used to investigate cell lineage and cell division patterns in leaves (Steffensen, 1968; Poethig and Sussex, 1985b; Langdale et al., 1989; Irish and Sussex, 1992; Poethig and Szymkowiak, 1995).

### 1.4.2 Development of dicot leaves

Dicot leaves are initiated by periclinal divisions in a subset of cells in the peripheral zone of the SAM. The first periclinal divisions are generally in the subsurface layers, and are followed by anticlinal divisions in the surface layer (Esau, 1960). These divisions result in a bulge on the flank of the SAM, referred
to as the leaf buttress. The leaf primordium emerges from the leaf buttress as a peg-like outgrowth. Esau (1960) refers to this phase as formation of the leaf axis. At this stage there is no distinction between the blade and petiole (Poethig and Sussex, 1985a). During this phase, the procambium is differentiated in continuity with the procambium of the internode below. The lamina is initiated from cells at the boundary between the adaxial and abaxial faces of the primordium (Poethig and Sussex, 1985a). Subsequent lateral growth of the lamina gives the leaf obvious medial-lateral, and adaxial-abaxial axes (Esau, 1960). Lateral veins are initiated at an oblique angle to the midrib (Poethig and Sussex, 1985a).

A series of classic surgical experiments provided evidence that communication between the SAM and developing leaf primordia is required for the establishment of adaxial cell identity, and for subsequent lamina outgrowth (Sussex, 1955; Snow, 1959). Sussex (1955) found that leaf anlagen (incipient leaf primordia) isolated from the shoot apex by tangential incisions developed as radially symmetrical, abaxialised organs. Leaf primordia isolated after emergence developed flattened laminae. Sussex proposed that communication between the shoot apex and developing leaf primordia is required for the development of adaxial features and lateral growth. These experiments imply that adaxial and abaxial domains are established during the transition from anlagen to leaf primordium. Furthermore, they suggest that abaxial identity is the default state when the leaf primordium is deprived of positional cues.

1.4.3 Development of maize leaves
The maize leaf is derived from cells of the two outer layers of the SAM, the L1 and L2 (Sharman, 1942; Poethig, 1984). Periclinal cell divisions are initiated in a position that corresponds to the future midrib, and are propagated laterally in both directions until they encircle the SAM. The leaf primordium appears first as a crescent shaped protrusion and then as a ridge of cells that encircles the apex (Sharman, 1942; Esau, 1965). Vascularisation begins with the acropetal (base to tip) development of the midvein into the P0 or P1 primordium. This occurs in continuity with the procambium of the shoot apex (Sharman, 1942).
Sylvester et al. (1990) have defined three phases of leaf development following emergence of the leaf primordium. Initially, cell division is even throughout the leaf primordium and the sheath and blade regions are morphologically indistinguishable. Lateral veins differentiate acropetally during this primordial stage, beginning near the midrib (Sharman, 1942).

During the second stage, a localised increase in cell division rate, accompanied by a decrease in cell extension, generates a band of small cells (the preligule band) near the base of the leaf primordium. Formation of the preligule band separates the future blade and sheath. Periclinal divisions by epidermal cells at the base of the preligule band form the ligule, and the remaining cells of the preligule band form the auricle. During this phase, cell divisions in the leaf blade become exclusively transverse and differentiation begins at the tip of the blade. Intermediate veins differentiate basipetally, subdividing the regions between the laterals. Transverse veins develop and connect the system of longitudinal veins (Sharman, 1942). During the third stage, the sheath grows rapidly. Differentiation proceeds basipetally, with the sheath being the last part of the leaf to differentiate (Sharman, 1942).

1.5 Developmental compartments and axial patterning

1.5.1 Developmental compartments

A primary mechanism in animal development is the subdivision of fields of cells into progressively smaller compartments (Lawrence and Struhl, 1996). Compartments are defined by differential gene expression. New axes may form when two compartments are juxtaposed. In Drosophila limb development, the boundaries between anterior and posterior, and dorsal and ventral, compartments act as organising centres. Experiments show that generation of ventral cell-types in the dorsal compartment creates an ectopic boundary, resulting in the formation of a secondary axis (Diaz-Benjuemea and Cohen, 1993). There is evidence for similar mechanisms in leaf development, although compartment boundaries are less rigid. Analysis of genetic mosaics in Drosophila demonstrates that cells do not divide across compartment boundaries (Garcia-Bellido et al., 1973). In plants, developmental compartments are more plastic. Cells may divide into adjacent compartments, and
subsequently differentiate according to position rather than lineage (Steeves and Sussex, 1984).

1.5.2 Analysis of leaf patterning mutants
Analysis of mutants that show defects at different stages of leaf development provide clues about the genetic pathways that regulate normal development. Maize is a useful model for such investigations as it is amenable to genetic analysis. Many mutants have been described and the leaves are large, with distinct boundaries and cell types distinguishing different compartments (Freeling and Hake, 1985; Freeling, 1992). Information about the normal role of a gene may be obtained by comparisons of normal and mutant morphogenesis, and of mature organ phenotypes (Sylvester et al., 1990). Interactions between mutants can help determine their order in genetic pathways, whilst analysis of dominant mutations allows identification of genes that may be obscured by genetic redundancy in single loss-of-function mutants (Freeling and Fowler, 1994). Mapping and cloning of genes opens the way for further functional analyses such as studies of gene expression in wild-type and in other mutant backgrounds.

1.5.3 Proximal-distal patterning
The maize leaf is comprised of a proximal sheath and distal blade, separated by a fringe of ligule tissue and two wedges of auricle tissue. Mutants that affect proximal-distal patterning of the maize leaf include dominant Knox mutants and Wab1 mutants (Sinha and Hake, 1990; Hay and Hake, 2004). The liguleless (Ig) mutants, Ig1 and Ig2, remove ligule and auricle tissue, but maintain a boundary between blade and sheath (Emerson, 1912; Brink, 1933). Although dominant Knox mutations disrupt proximal-distal patterning, their normal function is not specification of the blade-sheath boundary.

Dominant Knox mutations
In maize, a number of dominant Knox mutations disrupt proximal-distal patterning of the leaf (Sinha and Hake, 1990; Becraft and Freeling, 1994; Foster et al., 1999). The mutants are characterised by the development of proximal tissue types, such as sheath and auricle, in the leaf blade. Knotted1 (Kn1)
mutants were the first of these to be identified. Cloning of dominant Kn1 alleles revealed that mutations in non-coding regions are responsible for ectopic expression of kn1 (Hake et al., 1989; Veit et al., 1990). In situ hybridisation and immunolocalisation have shown that the wild-type gene (kn1) is expressed in the SAM, and is downregulated in incipient leaf primordia (Smith et al., 1992). The region of knox downregulation correlates with estimates of founder cell populations obtained from clonal analysis. In Kn1 mutants, the gene is ectopically expressed in the vascular bundles of developing leaves. Based on these results, it is hypothesised that the normal function of kn1 is to maintain cells in a meristematic state, and that ectopic expression delays differentiation, thus altering cell fates (Smith et al., 1992).

Knox genes appear to have similar functions in dicots and grasses. SHOOTMERISTEMLESS (STM), the kn1 homologue in Arabidopsis, has an expression pattern that is comparable to kn1. Loss-of function stm mutations result in embryos that lack SAMs, whereas overexpression of Knox genes is associated with less determinate patterns of growth, such as lobed leaves and ectopic meristem formation (Barton and Poethig, 1993; Sinha et al., 1993; Chuck et al., 1996).

Rough sheath2 (Rs2) negatively regulates knox expression in maize. rs2 loss-of-function mutants ectopically express knox genes and have phenotypes similar to Knox misexpression (Schneeberger et al., 1998). Ectopic knox expression in rs2 mutants is patchy and appears clonal in nature (Timmermans et al., 1999). There is evidence that Rs2 and the Arabidopsis orthologue, ASYMMETRIC LEAVES1, act as epigenetic regulators of knox genes, possibly by modifying chromatin structure (Phelps-Durr et al., 2005).

Freeling (1992) has proposed a "maturation schedule" hypothesis to account for dominant Knox phenotypes. These mutations cause proximal tissues to extend into the blade, and immature cell types are often associated with regions of displaced tissue (Freeling and Hake, 1985). Freeling (1992) speculates that the ectopic tissues are the result of a retarded maturation schedule. According to this model, ectopic expression of Knox genes delays the maturation of cells. Affected cells are not competent to respond to signals to differentiate at the time
when the rest of the blade is differentiating. Instead, they differentiate as the more juvenile sheath tissue.

Although proximal-distal patterning is disrupted by dominant Knox mutations, the normal role of these genes is not to specify blade or sheath compartments, nor to demark a boundary between these regions. In wild-type maize, knox genes are not expressed in the leaf primordium. Rather, their normal role seems to be to define a "meristematic domain".

**Wab1-R causes proximal-to-distal tissue displacement**

In maize, dominant Wab1 mutations cause displacement of proximal tissues such as sheath and auricle into the leaf blade (Hay and Hake, 2004). In addition, the leaf blade is narrower than normal. This defect is apparent by P3-P5 (i.e. in leaf primordia that are third to fifth from the SAM). Although the phenotype is similar to dominant Knox mutations, Wab1 mutants show no alteration in knox expression (Hay and Hake, 2004).

**liguleless1 and liguleless2 delete ligule and auricle tissue**

Lg1 and Lg2 act in a pathway that specifies ligule and auricle tissues. Loss of either gene function results in deletion of these tissues. However, leaves retain distinct regions of blade and sheath (Becraft et al., 1990; Harper and Freeling, 1996). Lg1 encodes a novel protein that localises to the nucleus (Moreno et al., 1997). Lg2 encodes a basic leucine zipper protein (Walsh et al., 1997). Mosaic analysis indicates that Lg1 has a role in signal propagation, and acts cell-autonomously to specify ligule and auricle tissues (Becraft et al., 1990; Becraft and Freeling, 1991). Lg2 acts non-cell autonomously (Walsh et al., 1997). Sylvester et al. (1990) have analysed cell division in lg1 mutants. They found that the localised cell divisions that normally give rise to the preligule band are absent in lg1 leaves.

Mosaic analyses have been used to investigate the roles of Lg1 and Lg2 in maize leaf development. Mosaic analysis combines clonal analysis and analysis of mutant phenotypes. The technique involves the generation of organisms that are genetic mosaics for a gene of interest and a linked marker gene. Mosaic analysis has shown that Lg1 acts cell-autonomously to specify ligule and auricle
tissues (Becraft et al., 1990). When Ig1-R/- sectors are induced in Lg1/Ig1-R plants, the ligule and auricle are deleted within the sector and reininitate at the marginal sector boundary. The reininitiated ligule and auricle are often displaced towards the base of the leaf (Becraft and Freeling, 1991). One interpretation is that Lg1 is involved in the reception and/or transmission of a “make ligule and auricle” signal that emanates from the midrib towards the margins. As differentiation proceeds basipetally, the time taken for the signal to cross the Ig1-R/- sector means that the tissue directly opposite the initiation point is no longer competent to respond to the signal. Cells in more proximal positions respond, resulting in displacement of the ligule and auricle at the outer sector border (Becraft and Freeling, 1991).

1.5.4 Adaxial-abaxial patterning

Perhaps the most compelling example of compartments in leaf development is adaxial-abaxial patterning. In dicots, the leaf primordium emerges as a radial or peg-like outgrowth and polarity genes are expressed uniformly. As the primordium emerges, adaxial and abaxial patterning genes become confined to their respective domains (Sawa et al., 1999; Siegfried et al., 1999; Kerstetter et al., 2001; McConnell et al., 2001). One theme that has emerged from mutant studies is that the juxtaposition of adaxial and abaxial compartments is required for subsequent lamina outgrowth (Waites and Hudson, 1995). It is hypothesised that signalling at the boundary between the two domains creates a lateral axis, and in some way induces growth along this axis. Complete loss of either adaxial or abaxial identity results in the formation of radialisled lateral organs. Conversely, when ectopic patches of either adaxial or abaxial tissue occur next to tissue with the opposite identity, ectopic outgrowths occur. It is proposed that the adaxialising signal originates in the centre of the SAM (McConnell et al., 2001). These studies are consistent with, and extend, classic surgical investigations of leaf development (Sussex, 1955; Snow, 1959).

phantastica and the juxtaposition model

The juxtaposition hypothesis for lamina development was inspired by the phantastica (phan) loss-of-function phenotype in Antirrhinum (Waites and Hudson, 1995). phan mutants exhibit a range of phenotypes including radial
leaves with abaxial cell types, and leaves with outgrowths at the boundaries between adaxial and ectopic abaxial tissues. It was hypothesised that signalling between adjacent adaxial and abaxial domains induces lamina outgrowth.

The *phan* phenotype was initially characterised as a loss of adaxial identity. An alternative explanation has been offered by Tsiantis and co-workers (1999) who propose that *phan* is primarily a proximal-distal patterning defect, with the radialised leaves resulting from extension of the proximal petiole. This hypothesis will be discussed further with reference to the maize homologue *Rough sheath2* (*Rs2*). Nonetheless, the juxtaposition model has provided a key to interpreting the phenotypes of other mutants that affect adaxial-abaxial polarity.

**HD-ZIPIII genes specify adaxial cell fate**

Genetic analyses indicate that the *Class III homeodomain-leucine zipper (HD-ZIPIII)* genes specify adaxial identity in lateral organs in dicot species and in maize. HD-ZIPIII proteins have a homeodomain-leucine zipper domain and a START domain similar to mammalian sterol/lipid-binding proteins (Sessa *et al.*, 1998; Pontig and Aravind, 1999; McConnell *et al.*, 2001; Juarez *et al.*, 2004a).

Dominant mutations in the *Arabidopsis HD-ZIPIII* genes, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), result in their ectopic expression and adaxialisation of lateral organs (McConnell and Barton, 1998; McConnell *et al.*, 2001). REV mutants have an additional phenotype – the development of radial, adaxialised vascular bundles in the stem (Emery *et al.*, 2003). Triple loss-of-function mutants have abaxialised cotyledons, and the SAM fails to form. Vascular bundles in the cotyledons are radialised, with phloem surrounding xylem (Emery *et al.*, 2003).

Expression studies show that *PHB, PHV* and *REV* are initially expressed throughout leaf anlagen, and become localised to the adaxial domain by the P2 stage of leaf development. They are also expressed in the central part of the embryo from the globular stage, and in xylem of developing vascular bundles (McConnell *et al.*, 2001; Emery *et al.*, 2003). In dominant mutants, *HD-ZIPIII*
genes are expressed in both the adaxial and abaxial domains and are expressed at higher levels than in wild-type (McConnell and Barton, 1998; McConnell et al., 2001). The expression patterns, along with the mutant phenotypes suggest multiple roles in patterning the embryo, lateral organs and vasculature (McConnell et al., 2001; Emery et al., 2003).

Dominant mutations in a maize HD-ZIPIII gene, rolled leaf1 (rld1), also affect adaxial-abaxial polarity. rld1 is the maize homologue of REV (Juarez et al., 2004b). Dominant Rld1 mutants show partial to complete switching of adaxial and abaxial epidermal features in sectors of the leaf (Figure 1.4 B) (Nelson et al., 2002). These switches involve adaxialisation of abaxial regions, such as ligule flaps on the abaxial surface, or a complete switching of epidermal features.

Like the Arabidopsis HD-ZIPIII genes, rld1 and a maize PHB homologue are normally expressed at the tip of the SAM and on the adaxial side of incipient and young leaf primordia (Juarez et al., 2004b). Expression persists in the vasculature and at the margins. The dominant Rld1-O allele is misexpressed on the abaxial side of leaf primordia, although expression in the meristem is not altered.
**Regulation of HD-ZIPIII genes by microRNAs**

McConnell and co-workers (2001) proposed that PHAB acts as a receptor for an adaxialising signal that originates in the centre of the SAM. Dominant alleles have mutations in the START domain and initially it was thought these mutations caused the protein to be constitutively active in the absence of ligand binding (McConnell et al., 2001). However, subsequent investigations indicate that this domain overlaps with a miRNA-complementary region and that ectopic expression is due to loss of regulation by the miRNAs miR165 and miR166 (Rhoades et al., 2002; Emery et al., 2003). Similarly, dominant Rld1 alleles in maize have nucleotide changes in the miRNA-complementary region, indicating that regulation by miRNA165/166 is a conserved mechanism (Juarez et al., 2004b). In maize, miRNA166 is expressed immediately below the incipient leaf primordium. Subsequently, the expression domain expands to include the abaxial side of the primordium (Juarez et al., 2004b).

These findings do not preclude a role for a ligand that activates HD-ZIPIII proteins in adaxial regions of lateral organs. Regulation by miRNAs and a ligand produced in the SAM could act together to specify and refine the domain of active HD-ZIPIII protein (Emery et al., 2003; Juarez et al., 2004b). Juarez et al. (2004b) suggest that rld1 may act to integrate positional information from the SAM and from below the leaf primordium.

**KANADI genes specify abaxial cell fate**

Members of the *Arabidopsis KANADI* (KAN) gene family are expressed abaxially in lateral organs, and analysis of loss-of-function and overexpression phenotypes indicate that they are required for abaxial cell identity. KAN genes belong to the GARP family of transcription factors (Kerstetter et al., 2001).

The KAN family has four members in *Arabidopsis* (Kerstetter et al., 2001; Eshed et al., 2004). Due to genetic redundancy, loss of several genes is required to generate the most severe phenotypes (Eshed et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001). kan1 and kan1;kan2 mutant floral organs are adaxialised, and leaves are narrow with ectopic outgrowths on their abaxial
surface associated with patches of adaxial tissue (Eshed et al., 2001; Kerstetter et al., 2001). When kan1, 2 and 3 functions are lost, outgrowths are reduced and leaves are nearly radial. Vascular bundles in the stem are often radialised with xylem surrounding phloem (Emery et al., 2003; Eshed et al., 2004). Seedlings that overexpress KAN genes often lack a SAM and vascular tissue, and have a single abaxialised cotyledon. If leaves are produced, they are narrow or radial (Kerstetter et al., 2001; Emery et al., 2003).

KAN1, 2 and 3 are expressed in all lateral organs, while KAN4 expression is limited to the ovules (Kerstetter et al., 2001; Eshed et al., 2004). KAN genes are initially expressed throughout the leaf anlagen, and become confined to the abaxial domain after emergence of the primordium (Kerstetter et al., 2001). KAN1, 2 and 3 are expressed in developing vasculature and localise to the phloem (Emery et al., 2003). KANs are expressed throughout the early globular stage embryo, before becoming confined to the peripheral region by the late globular stage (Kerstetter et al., 2001).

Less is known about the KAN family in maize. There are at least 11 KAN family members in maize (pers. comm., Hector Candela and Sarah Hake). Therefore, there is likely to be a high level of functional redundancy. ZmKAN2, the maize homologue of Arabidopsis KAN2, is expressed on the abaxial side of young leaf primordia in a pattern complementary to rld1. It is also expressed in the peripheral zone of the SAM and in the stem at the base of the P1 primordium (Henderson et al., 2006). The expression pattern in leaf primordia is similar to the KAN expression pattern in Arabidopsis. However, Arabidopsis KANs are not expressed in the SAM or the stem. Therefore, there may be functional differences in maize and Arabidopsis. Henderson et al. (2006) state that while the abaxial expression pattern is consistent with a role in promoting abaxial identity as in Arabidopsis, this is speculative as no mutant phenotypes have yet been analysed.

Recent data indicate that the KAN genes may act in a parallel pathway with the auxin response factors ETTIN (ETT) and Auxin Response Factor4 (ARF4) (Pekker et al., 2005). These genes are expressed on the abaxial side of leaf primordia and in kan1;kan2 outgrowths. ett;arf4 loss-of-function mutants have
adaxialised leaves, similar to kan loss of function phenotypes. The data indicate that ETT and ARF4 do not act downstream of KAN, but in a parallel pathway. It is proposed that these genes are expressed in the abaxial domain in response to auxin gradients in the primordium. Recent research has begun to elucidate many other interactions between plant hormones and genes that regulate organ development (Kepinski, 2006).

**Interactions between HD-ZIPIII and KANADI genes**

Genetic interactions indicate that HD-ZIPIII and KAN genes act antagonistically to establish adaxial and abaxial domains. REV gain-of-function alleles, and loss of KAN activity have similar phenotypes, characterised by adaxialised lateral organs and altered vascular patterning. Conversely, phb;phv;rev loss-of-function mutant embryos lack a SAM and, in severe cases, have a single radial, abaxialised cotyledon (Emery et al., 2003). Overexpression of KAN causes a similar phenotype (Eshed et al., 2001; Kerstetter et al., 2001). HD-ZIPIII and KAN genes normally have complementary expression patterns. Loss of KAN activity results in expansion of the HD-ZIPIII expression domain (Eshed et al., 2001). It is not clear whether the KAN genes specify abaxial cell types directly, or if their primary role is to repress HD-ZIPIII expression in abaxial domains (Emery et al., 2003). Based on the complementary expression patterns and mutant phenotypes of KAN and HD-ZIPIII genes, it has been proposed that a common mechanism patterns the central-peripheral axis of the embryo and the adaxial-abaxial axis of lateral organs and vasculature (McConnell et al., 2001; Emery et al., 2003).

**leafbladeless1 is required for founder cell recruitment**

Analysis of recessive leafbladeless1 (lbl1) mutants in maize suggests that adaxial-abaxial polarity is established within the SAM, and is required for the lateral propagation of founder cell recruitment (Timmermans et al., 1998). Severe lbl1 mutants have extremely narrow, abaxialised leaves. The needle-shaped leaves usually have a narrow ligular fringe and two rows of marginal hairs in close proximity, suggesting a severely reduced adaxial domain (Figure 1.4 C). Immunolocalisation of KN1 protein shows the region of KN1 downregulation is significantly reduced in severe lbl1 mutants, implying that
founder cell recruitment is defective. A less severe phenotype involves the formation of ectopic laminae on the adaxial surface of the leaf blade (Figure 1.4 D). These ectopic outgrowths form at the boundary of adaxial tissue and abaxialised mutant sectors. The phenotypes suggest that the juxtaposition of adaxial and abaxial cell types is required for the lateral recruitment of founder cells within the SAM, and for subsequent lateral growth of the leaf blade.

The Rld1 and Ibl1 phenotypes are mutually suppressive (Juarez et al., 2004a). rld1 expression is reduced in Ibl1 mutants, indicating that Ibl1 acts upstream of rld1 (Juarez et al., 2004a). It has emerged recently that Ibl1 acts in a pathway that produces a regulatory RNA which in turn regulates MiR166. Higher levels of MiR166 in Ibl1 mutants would account for the lower levels of rld1 expression in these mutants (per. comm. Marja Timmermans).

**YABBY genes promote lamina outgrowth**

Members of the YABBY (YAB) gene family are associated with abaxial cell fate in Arabidopsis. Multiple lines of evidence support a role for the YABs in lamina outgrowth in maize and in dicot species.

The Arabidopsis YABBY family comprises 6 members (Bowman and Smyth, 1999; Eshed et al., 1999; Sawa et al., 1999; Siegfried et al., 1999). YAB proteins have a zinc finger-like domain towards the amino terminus and a helix-loop-helix domain, known as the YABBY domain, near the C-terminus (Bowman and Smyth, 1999). Due to genetic redundancy, loss of several genes is required to see a loss-of-function phenotype. Overexpression causes the development of abaxialised lateral organs and meristem arrest, whereas loss of YAB function results in adaxialised organs (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Eshed et al., 1999; Sawa et al., 1999; Siegfried et al., 1999; Villanueva et al., 1999). Loss of YAB function is associated with misexpression of KNOX genes and the formation of ectopic meristems on the adaxial side of leaves and cotyledons, suggesting an additional role in repressing meristem initiation (Kumaran et al., 2002).
Genetic and molecular data indicate that YAB function is downstream of KAN in Arabidopsis. YAB genes are expressed in a similar pattern to the KANs. They are initially expressed throughout lateral organ anlagen, and become confined to the abaxial side of the primordium after emergence (Siegfried et al., 1999). FILAMENTOUS FLOWER (FIL) is expressed throughout leaf primordia that ectopically express KAN2, although expression is transient (Eshed et al., 2004). YAB activity is both necessary and sufficient for the development of abaxial cell types in a kan loss-of-function background (Eshed et al., 2001; Eshed et al., 2004).

GRAMINIFOLIA (GRAM) is the Antirrhinum orthologue of FIL (Golz et al., 2004). gram leaves and petals are partially adaxialised, particularly at the margins, and are narrower than normal. In many cases, the corolla tube is unfused. GRAM appears to promote abaxial fate by excluding adaxial factors such as AmPHB, a function performed by KAN genes in Arabidopsis. In addition, there is evidence that GRAM promotes adaxial cell identity non-autonomously. Golz et al. (2004) propose that the primary function of GRAM may be to refine and maintain the boundary between adaxial and abaxial domains, and that this boundary is required for lateral growth. Unlike many other leaf polarity mutants, no outgrowths are seen at ectopic adaxial-abaxial boundaries in gram leaves.

The maize Zea mays yabby (Zyb) genes are expressed on the adaxial side of incipient and young leaf primordia, opposite to the pattern seen in Arabidopsis (Juarez et al., 2004a). In older leaf primordia, expression persists at the margins and in the central layer. Expression of Zyb9, but not Zyb14, persists in the vasculature. YAB expression is increased in Rld1-O, suggesting that YAB genes are positively regulated by rld1 in maize (Juarez et al., 2004a).

Various lines of evidence suggest that the YABs are required for lamina outgrowth. Loss of YAB function in Arabidopsis and Antirrhinum results in reduced lamina outgrowth, and ectopic outgrowths in kan mutants are dependant on YAB function (Siegfried et al., 1999; Eshed et al., 2004; Golz et al., 2004). In Arabidopsis, FIL is expressed in kan outgrowths (Eshed et al., 2004). It has been suggested that boundaries between YAB-expressing and
non-expressing cells promote outgrowth rather than absolute levels of YAB activity (Siegfried et al., 1999; Eshed et al., 2004; Golz et al., 2004). This function appears to be conserved in maize leaf development (Juarez et al., 2004a). In maize, Zyb expression is associated with abaxial outgrowths in Rld1-O leaves and adaxial outgrowths in lbl1. The Rld1-O outgrowths occur at the boundaries of Zyb-expressing and non-expressing cells of the central layer. It is suggested that polarised YAB expression may mediate founder cell recruitment in the maize SAM prior to emergence (Juarez et al., 2004a).

Genetic interactions that regulate adaxial-abaxial polarity

Figure 1.5 summarises interactions between leaf polarity genes discussed in this section. In Arabidopsis, KAN and HD-ZIPIII genes interact antagonistically to specify adaxial and abaxial domains in lateral organs. The expression pattern of HD-ZIPIII genes and a maize KAN2 homologue suggests a similar mechanism exists in maize, but this has not been proven.

Based on surgical experiments, Sussex (1955) proposed that communication between the SAM and lateral organ primordia is required for adaxial cell identity. The HD-ZIPIII proteins are candidate receptors for such a signal, although a ligand has not been identified. In both maize and Arabidopsis, HD-ZIPIII genes are regulated by miRNAs. It has been suggested that the maize HD-ZIPIII gene rld1 could integrate a signal from the SAM and miRNA signalling from below the leaf primordium (Juarez et al., 2004a). Recent data indicate that miRNA166 in maize is regulated by a second miRNA, and that lbl1 is involved in this pathway.

The HD-ZIPIII genes are associated with adaxial cell fate in both Arabidopsis and maize. A role for the maize KAN genes in promoting abaxial cell fate is speculative at this stage. The Arabidopsis KAN genes are associated with abaxial identity, but appear to act by promoting YAB expression, rather than specifying abaxial cell fate directly. In Arabidopsis, the YABs are expressed abaxially, downstream of the KANs, and specify abaxial cell identity. In contrast, the maize YAB genes are expressed adaxially and are downstream of rld1.
(Juarez et al., 2004a). In both maize and Arabidopsis, the YABs are implicated in lateral growth, and may promote founder cell recruitment in maize.

Figure 1.5. Interactions between factors involved in the specification of adaxial-abaxial polarity in Arabidopsis and maize leaves.

**1.5.5 Medial-lateral patterning**

**Narrow sheath** function is required for propagation of founder cell recruitment

Analysis of *narrow sheath* (*ns*) mutants provides evidence for the existence of at least two lateral domains in the maize leaf – a marginal domain defined by *ns*, and a central domain. *NS1* and *NS2* are duplicate genes. Plants that are homozygous for both *ns1* and *ns2* have narrow leaves that lack the marginal characteristics of normal leaves (Figure 1.4 E). Cell lineage analysis indicates that a lateral domain is deleted. Albino sectors that in wild-type leaves would continue into the leaf margins are confined to the culm in *ns* plants (Scanlon and Freeling, 1997). Immunolocalisation of KN1 within the SAM has confirmed that KN1 is not downregulated in the domain that would normally give rise to the leaf margins (Scanlon et al., 1996). In normal development, founder cell recruitment begins at the future midrib, and is propagated laterally to the marginal domains (Jackson et al., 1994). A mosaic analysis has shown that loss of NS function at focal points on either side of the SAM results in the development of margin-less leaves (Scanlon, 2000). When NS is lost in other regions, leaves develop normally. *In situ* hybridisation has confirmed that NS is
expressed in two lateral foci in leaf founder cells, and also at the margin tips of leaf primordia (Nardmann et al., 2004). These results suggest that NS function in lateral foci is required for the propagation of founder cell recruitment to the marginal domains. The central domain is initialised by another, as yet unknown, mechanism.

1.5.6 Summary of axial patterning

Analysis of leaf patterning mutants demonstrates that disruption of one axis affects specification of the other axes, indicating that the major axes of growth are not established independently. For example, disrupting adaxial-abaxial polarity affects establishment of the medial-lateral axis. The phenotypes of mutants that fail to establish either adaxial or abaxial cell identity support the hypothesis that the juxtaposition of these two compartments establishes the medial-lateral axis. There is evidence to support this hypothesis in both dicot and maize leaf development. However, there are also fundamental differences in leaf morphogenesis. In dicots, adaxial and abaxial domains are established after founder cell recruitment. In maize, adaxial and abaxial domains and the medial-lateral axis are established in the SAM, and are required for the lateral propagation of founder cell recruitment.

Members of the KAN, YAB and HD-ZIPIII families are found in monocots and dicots (Floyd and Bowman, 2007). However, their functions and interactions have diverged somewhat. The Arabidopsis YABs are expressed abaxially and are downstream of KAN, whereas the maize YABs are expressed adaxially and are regulated by HD-ZIPIIIIs. It has been suggested that the specification of abaxial identity that is fulfilled by YAB genes in Arabidopsis may be performed by KAN genes in maize (Juarez et al., 2004a). A common function of YAB genes in Arabidopsis, Antirrhinum and maize appears to be promotion of lateral growth. In Antirrhinum, YAB genes may inhibit members of the HD-ZIPIII family, a role performed by the KAN genes in Arabidopsis. Thus, it is important not to assume orthologous functions without functional data.
1.6 Establishment and maintenance of developmental domains

During development, groups of cells are partitioned for particular fates. For example, cells that will be incorporated into a lateral organ must be distinguished from cells that will remain meristematic. Cells that will form the sheath or petiole must be distinguished from those that will form the leaf blade. However, to ensure coordinated development there must be continued communication between groups of cells. There is a network of transcriptional activators and repressors that control gene expression. The previous section focused on the importance of domains of gene expression in leaf patterning, and the consequences of disrupting this positional information. In addition to transcriptional regulation, there are post-transcriptional mechanisms that act to establish and maintain developmental domains. Two such mechanisms are the post-transcriptional regulation of gene expression by miRNAs, and the regulation of protein movement between cells.

1.6.1 MicroRNAs and gene regulation

Recent work has revealed the role of miRNAs as negative regulators of gene expression. miRNAs are short RNAs transcribed from non-protein coding genes that act as specificity determinants within complexes that target mRNAs for degradation (Carrington and Ambros, 2003). Information has come from studies of mutations that disrupt miRNA-mediated regulation, such as dominant HD-ZIPIII mutants, and from mutations to genes encoding proteins that form part of the miRNA processing machinery. MiRNA targets include families of transcription factors with roles in development in both plants and animals (Carrington and Ambros, 2003).

In plants, disruption of miRNA-mediated gene silencing produces a variety of defects, including leaf patterning defects that resemble dominant HD-ZIPIII mutations (Foster et al., 2002). ARGONAUTE1 (AGO1) functions in the miRNA pathway, and ago1 mutants have leaf polarity defects (Carrington and Ambros, 2003; Kidner and Martienssen, 2004). In ago1 mutants, PHB is expressed throughout the leaf primordia (Kidner and Martienssen, 2004).
The HD-ZIPIII genes \textit{PHB}, \textit{PHV} and \textit{REV} contain a conserved complementary site for miRNA165/166 which can direct their cleavage \textit{in vitro} (Tang \textit{et al.}, 2003). Complementary sites overlap the sites of mutations in dominant alleles that are ectopically expressed. Experiments show that changing the \textit{REV} mRNA sequence without changing the protein results in a gain-of-function phenotype. It was concluded that this phenotype results from interference with miRNA binding (Emery \textit{et al.}, 2003). There is evidence that miRNAs also regulate \textit{HD-ZIPIII} genes in differentiated cells by mediating chromatin methylation (Bao \textit{et al.}, 2004).

1.6.2 Protein trafficking

Cells must communicate with their neighbours to ensure coordinated cell division and differentiation, necessitating the exchange of signalling molecules. The observation that cells differentiate according to position rather than lineage implies that positional information is communicated between neighbouring cells. One method is the movement of proteins from cell to cell through the plasmodesmata. Dynamic regulation of the plasmodesmata occurs during development. Evidence for symplastic domains included observations of the cell-to-cell trafficking of dyes, viral movement proteins and green fluorescent protein (GFP) (Gisel \textit{et al.}, 1999; Crawford and Zambryski, 2000). It was found that proteins could move freely between some cells, but were excluded from others. Recent studies indicate that the passage of specific proteins is under developmental control (Kim \textit{et al.}, 2003).

Mosaic analysis of a number of developmentally important genes indicates that they may act in a non cell-autonomous manner. A mosaic analysis of \textit{Kn1} found that \textit{Kn1} expression in the mesophyll was sufficient to condition the mutant phenotype in all cell layers, whereas expression in the L1 alone did not condition the mutant phenotype (Hake and Freeling, 1986). This result implied that either the gene product itself, or some downstream mediator(s), are able to move cell-to-cell. \textit{In situ} hybridisation and immunolocalisation confirm that while \textit{kn1} mRNA accumulates in the L2 and L3, but not the L1, KN1 protein accumulates in all cell layers (Smith \textit{et al.}, 1992; Jackson \textit{et al.}, 1994). Similarly, expression of the \textit{Arabidopsis} floral meristem identity gene \textit{LEAFY}
(LFY) in just the L1 of *lfy* mutants can rescue the *lfy* mutant phenotype in all cell layers. While *LFY* mRNA accumulated only in the L1, LFY protein was seen in all cell layers. This finding, as well as confirmation that transported LFY can activate downstream genes, indicates that the protein retains its biological activity (Sessions *et al*., 2000).

Exclusion of KN1 from incipient leaf primordia implies that protein movement within the SAM is developmentally regulated. Similarly, there is a sharp boundary of LFY protein between floral primordia and the inflorescence meristem, suggesting that protein movement across this boundary is blocked (Sessions *et al*., 2000). The restriction of protein movement may function to partition groups of cells with different fates. Conversely, protein trafficking between cells with a common fate could ensure that all cells within a developmental field are synchronised to a given programme. For example, this could ensure that floral meristems undergo complete conversion to floral identity, and do not produce chimeric organs (Sessions *et al*., 2000). KN1 trafficking within the SAM may ensure that all cells retain their meristematic identity (Kim *et al*., 2003).

The initiation of organs by the SAM is a dynamic process. It follows that the regulation of developmental domains must also be dynamic. Experiments using KNOX proteins fused to GFP (GFP~KN1) or viral movement proteins demonstrate that trafficking of KNOX proteins is developmentally regulated in *Arabidopsis* (Kim *et al*., 2003). GFP~KN1 could traffic from the inner layers of the leaf to the epidermis (L1), but not in the opposite direction, but could traffic out of the L1 in the SAM. Unlike the GFP~KN1 fusion, GFP~movement protein could move out of the leaf epidermis, indicating that regulation was proteinspecific. The mechanism is not yet known, but could be mediated by receptors within the plasmodesmata that recognise specific motifs, by post-translational protein modifications, or by a combination of mechanisms.
1.7 Homologies between lateral organs

1.7.1 Plants are comprised of repeated structural units

Plants have a metameric organisation, with the phytomer considered to be the basic structural unit. Each phytomer consists of a node, internode and leaf (Weatherwax, 1923; Sharman, 1942). In grasses, the leaves are subdivided into blade and sheath. Morphological diversity is achieved by the differential elaboration or repression of developmental compartments (Galinat, 1959). This is apparent in comparisons of different species, and when comparing vegetative and floral organs of the same plant. Analysis of developmental mutants reveals that leaves and homologous organs are patterned by common genetic programmes, as well as organ-specific programmes (Bossinger et al., 1992).

According to the leaf zonation hypothesis, leaves are comprised of two morphological compartments along the proximal-distal axis – the upper and lower leaf zones (Figure 1.7) (Troll, 1955; Kaplan, 1973). Dicot leaves are derived mainly from the upper leaf zone, whereas, maize leaves are almost entirely lower leaf zone. Maize vegetative and husk leaves differ in the relative contributions of the blade and sheath (Figure 1.6 A, B). The blade is the dominant part in vegetative leaves, whereas the husk leaves are mainly sheath tissue.

One conserved element of phytomer organisation in the grasses is the production of fused organs by newly initiated meristems. Bossinger et al. (1992) termed the first phytomer produced by a newly initiated meristem a "type 2 phytomer". Subsequent phytomers each have a single leaf primordium and are termed "type 1 phytomers". The type 2 phytomer is typified by the prophyll which subtends the female inflorescence (the ear). The prophyll is believed to develop via the congenital fusion of the first two husk leaves along adjacent margins (Figure 1.6 C) (Bossinger et al., 1992; Scanlon and Freeling, 1998). Other organs that may be classed as type 2 phytomers are the coleoptile, palea, lodicules and stamens (Bossinger et al., 1992). The glumes are the first organs to be initiated by the spikelet axis. However, in maize and barley they are not fused organs. Bossinger et al. suggest that the two glumes may be
considered together to correspond to a type two phytomer, a suggestion that is supported by the finding that in some species the two glumes are fused.

The silks (gynoecia) are thought to represent a different form of phytomer fusion. According to one model, the silks develop via the congenital fusion of two carpels along two sets of margins (Cronquist, 1988; Scanlon and Freeling, 1998) (Figure 1.6 D).

Figure 1.6. Lateral organ homologies in maize. (A) Vegetative leaves are subdivided into blade (green) and sheath (blue). (B) Husk leaves are primarily sheath tissue with a small residual blade at the tip. (C) The prophyll is believed to develop via the congenital fusion of the first two husk leaves along adjacent margins. (D) The silks are thought to develop via the congenital fusion of two primordia along two sets of margins. (Cronquist, 1988; Bossinger et al., 1992; Scanlon and Freeling, 1998)
1.7.2 Mutant phenotypes provide a tool for detecting organ homologies within a species

Mutant phenotypes provide a useful tool for investigating homologies between lateral organs. For example, analysis of the ns phenotype provides support for the hypothesis that the maize prophyll is derived from two fused leaves (Scanlon and Freeling, 1998). The prophyll is the first organ to be produced by the newly initiated lateral meristem. It has two midribs (keels) separated by membranous tissue. In the ns mutant, the distance between the two keels is reduced, and the membranous tissue between the keels is replaced by thicker tissue. In addition, the outer margins are replaced by thick, blunt margins. Scanlon and Freeling (1998) propose that this is due to the deletion of the margins of each of the leaves that comprise the prophyll. The ns phenotype is consistent with the hypothesis that the prophyll is derived from two leaves fused along their inner margins, with each keel representing the midrib of a single leaf. In contrast, the palea (another organ with two midribs) is only affected at the margins while the distance between the midribs is not affected. Therefore, the ns phenotype provides no evidence that the palea is formed by phytomer fusion. The fused organ theory implies that the prophyll is derived from two founder cell populations. One way to test this would be to observe KNOX downregulation in lateral meristems during the earliest stages of prophyll initiation. Downregulation of KNOX proteins in two discrete spots would support the two phytomer theory.

1.7.3 Mutations to orthologous genes in diverse species

*rough sheath2* and *phantastica*

The homologous genes *PHAN* in *Antirrhinum*, and *Rs2* in maize negatively regulate *Knox* expression in lateral organ primordia and have loss-of-function phenotypes that are similar to overexpression of *Knox* genes (Waites and Hudson, 1995; Schneeberger *et al.*, 1998). Loss of *Knox* regulation in *rs2* mutants causes proximal-to-distal tissue displacement (Schneeberger *et al.*, 1998). Loss of function of *PHAN*, the homologous gene in *Antirrhinum*, causes upper leaves and floral organs to develop as radial, abaxialised structures (Waites and Hudson, 1995). This phenotype was originally interpreted as a defect in adaxial-abaxial patterning. However, in light of homology between *Rs2*
and PHAN, the *phan* phenotype has been interpreted by some authors as primarily a defect in proximal-distal patterning ("petiolization" of the leaf) rather than a defect in establishing adaxial-abaxial polarity (Tsiantis et al., 1999). In this interpretation, radialised *phan* leaves result from extension of the largely unifacial petiole region, equivalent to the extension of sheath tissue seen in *rs2* mutants. The fact that PHAN is not restricted to the adaxial domain, but is transcribed throughout the leaf primordium, is consistent with this model (Waites et al., 1998). This interpretation suggests that PHAN is the functional orthologue of *Rs2*.

**narrow sheath and pressed flower**

The leaf zonation hypothesis proposes that diverse leaf morphologies have evolved by the differential elaboration of upper and lower leaf zones (Troll, 1955; Kaplan, 1973). According to this model, bifacial monocot leaves such as maize are derived almost entirely from the lower leaf zone, whereas dicot leaves are derived mainly from the upper leaf zone (Figure 1.7). In maize, the lower leaf zone is highly elaborated, comprising the sheath and most of the blade, whereas the upper leaf zone is represented by only a small unifacial tip. In *Arabidopsis*, the lower leaf zone consists of the leaf base and stipules while the rest of the leaf is upper leaf zone. A comparison of the loss-of-function phenotypes of the homologous genes *ns* in maize, and *pressed flower (prs)* in *Arabidopsis* support this model (Nardmann et al., 2004).

![Figure 1.7. Upper and lower leaf zones.](image)

*Figure 1.7. Upper and lower leaf zones.* Upper and lower leaf zones in dicot (left) and monocot (right) leaves. Black indicates regions deleted by *prs* and *ns* mutations.

NS1, NS2 and PRS encode *WUSCHEL*-like homeobox genes (Matsumoto and Okada, 2001; Nardmann et al., 2004). NS1 and NS2 are expressed at two foci in the SAM, where they are required for recruitment of leaf founder cells in a lateral domain of the SAM. It is proposed that NS function is confined to the
lower leaf zone. Sequence identity, expression patterns, and mutant phenotypes suggest that ns and PRS are functional orthologues (Matsumoto and Okada, 2001; Nardmann et al., 2004). ns mutant leaves lack a marginal domain that encompasses most of the sheath and the lower leaf blade, whereas the leaf phenotype in Arabidopsis is restricted to deletion of the stipules at the base of the leaf (Figure 1.7) (Scanlon, 2000; Nardmann et al., 2004). It is suggested that the different phenotypes reflect differences in leaf morphogenesis in maize and Arabidopsis, specifically the different contributions of the upper and lower leaf zones, and the portion of the SAM that contributes founder cells to the leaf primordium (Scanlon, 2000). In maize, the lower leaf zone is highly elaborated and ns affects a large portion of the leaf. In contrast, the lower leaf zone is much reduced in dicot leaves and prs only affects the leaf base and stipules. The maize leaf founder cell population encompasses the entire circumference of the SAM. Therefore, the failure to initialise cells in a lateral portion of the SAM would be expected to delete a significant portion of the leaf. In contrast, Arabidopsis leaf founder cells occupy only a fraction of the circumference of the SAM and, as predicted, only a small portion of the leaf is deleted.

1.8 Genome duplication and subfunctionalisation

Polyploidy (whole genome duplication) is believed to have played an important role in plant evolution, particularly of flowering plants (Blanc and Wolfe, 2004; de Bodt et al., 2005; Duarte et al., 2006) There is evidence that maize is the descendant of an allotetraploid event that occurred around 4.8 million years ago (Gaut and Doebley, 1997; Swigonova et al., 2004). High levels of genetic redundancy are seen in maize as there are duplicate copies of most genes. It has been proposed that genome duplication provides opportunities for subfunctionalisation and neofunctionalisation (Lynch and Conery, 2000). A duplicated gene can have three possible fates – nonfunctionalisation (one copy becomes nonfunctional), neofunctionalisation (gain of a novel function) or subfunctionalisation (partitioning of the ancestral expression pattern between the two copies). Duplication events, followed by neofunctionalisation or subfunctionalisation, are believed to have contributed substantially to the evolution of morphological complexity (de Bodt et al., 2005).
Numerous examples of subfunctionalisation are evident in the families of transcription factors that regulate plant development. One example is the role of KAN genes in Arabidopsis ovule development (McAbee et al., 2006).

ABERRANT TESTA SHAPE (ATS, also known as KAN4) is expressed in the abaxial layer of the inner integument and is required for normal development of the inner integument. KAN1 and KAN2 act redundantly to provide a homologous function in the outer integument. The expression pattern parallels the expression of KAN genes in leaves. Thus, KAN family members have become specialised for very specific roles in ovule development.

1.9 Maize inbred lines

Maize is genetically very diverse, with a genome complexity comparable to that of humans (Tenailleon et al., 2001). Inbred lines are highly homogeneous, homozygous lines that have been created by multiple rounds of self-pollination and selection. They provide an array of uniform, reproducible genotypes that samples from this genetic diversity (Lee, 1994). Comparisons of inbred lines show high levels of sequence polymorphisms (Tenailleon et al., 2001; Fu and Donner, 2002; Song and Messing, 2003; Brunner et al., 2005). Much of this polymorphism is attributable to the activity of transposable elements (Lai et al., 2005; Morgante et al., 2005).

Studies have found differences in expression levels of homologous genes in different inbred lines (Song and Messing, 2003; Guo et al., 2004). A comparison of allelic chromosomal regions in two inbred line, Mo17 and B73, found extensive regions of non-homology (Brunner et al., 2005). It is proposed that the different sequence environment of conserved genes may affect their expression. Flanking sequences could influence gene expression by acting as enhancers, producing antisense transcripts, or by influencing chromatin state. These non-genic sequences could affect expression level, tissue specificity or temporal regulation of active genes.

It is useful to introgress into different inbred lines when characterising a mutant phenotype. Firstly, inbred lines provide uniform backgrounds in which to assess
the phenotype and, secondly, expression of many morphological traits varies greatly depending on background (Coe, 1994; Freeling and Fowler, 1994). This is due to the variability of modifying genetic factors in different backgrounds and differences such as developmental rate. Thus, introgressing into a range of inbreds can provide a spectrum of phenotypes for analysis. Suppression or alterations to mutant phenotypes in different genetic backgrounds can provide clues as to the function of the gene.

1.10 Aims and objectives

The overall aim of the project was to determine the roles of wab1 and Mwp1 in maize leaf development, and thus gain a better understanding of leaf axial patterning in grasses. Specific aims were as follows:

I. In order to test the hypothesis that Wab1-R acts cell-autonomously, and to extricate interactions between Wab1-R and Lg1, a mosaic analysis of Wab1-R was carried out in Lg1 and lg1-R backgrounds. To test the hypothesis that Lg1 acts cell-autonomously to condition ectopic auricle tissue in Wab1-R leaves and to promote lateral growth, a mosaic analysis of lg1-R was conducted in Wab1-R and wab1 backgrounds.

II. In order to gain a better understanding of the mechanisms controlling lateral growth of the maize leaf, the timing and location of the Wab1-R lateral growth defect were investigated by an analysis of vascular development in Wab1-R leaf primordia and a cell lineage analysis of Wab1-R leaves.

III. Characterisation of the mwp1-R phenotype and in situ hybridisation of known polarity genes were carried out in order to ascertain the role of Mwp1 in leaf development and to test the hypothesis that the mwp1-R mutation disrupts adaxial-abaxial polarity in lateral organs. To determine if mwp1-R affects lateral and proximal-distal growth of lateral organs, wild-type and mwp1-R leaves and floral organs were measured.
IV. In order to better understand the genetic control of axis specification in maize lateral organs with diverse morphologies, the polarity of *mwp1-R* and wild-type floral organs and prophylls were investigated by scanning electron microscopy (SEM) and light microscopy. Early prophyll development was characterised by SEM, and by *in situ* hybridisation of known polarity genes.
2. General Materials and Methods

2.1 Maize nomenclature

Symbols used to designate genes referred to in this study are in accordance with standard maize nomenclature (Burr et al., 1995).

Gene loci are represented by lower case italic characters. Where a mutant allele is recessive, it is designated by a lower case, italicised symbol. The symbol for dominant wild-type alleles is the same three letter symbol as for mutant alleles, but with the first letter capitalised. Where the mutant allele is dominant, the first letter of the symbol is capitalised. The symbol for the corresponding wild-type allele then has all lower case letters.

In maize, the first mutant allele discovered is referred to as the reference allele. Reference alleles are designated by "-R" after the three letter symbol. All mutant alleles used in this study were reference alleles. Thus, in this text the recessive reference allele at the \textit{mwp1} locus is referred to as \textit{mwp1-R} and the wild-type allele is referred to as \textit{Mwp1}. The dominant reference allele at the \textit{wab1} locus is referred to as \textit{Wab1-R}, and the wild-type allele is referred to as \textit{wab1}. The recessive reference allele at the \textit{lg1} locus is referred to as \textit{lg1-R}, and the wild-type allele is referred to as \textit{Lg1}.

Four marker genes were used for clonal and mosaic analyses. Recessive alleles of \textit{white seedling3-R (w3-R)}, \textit{lemon white-R (lw-R)} and \textit{albescent-R (al-R)} cause chlorophyll deficiencies and condition albino leaf phenotypes. The recessive allele \textit{virescent4-R (v4-R)} delays chlorophyll accumulation and conditions a yellow or virescent leaf phenotype.

2.2 Inbred lines

A number of standard maize inbred lines were used in this work. These were; A188, W23 and B73. These stocks were obtained from the Maize Genetics Coop.
2.3 Growth conditions

Field
The plants used in this study were grown in fields at the Institute of Developmental Phenomenology, Raumai, New Zealand and at the Massey University Plant Growth Unit, Palmerston North, New Zealand. Seeds were hand planted into cultivated soil in late November and grown to maturity.

Glasshouse
Seedlings for histology and SEM were grown in glasshouses at HortResearch, Palmerston North, New Zealand. Glasshouse temperature was maintained at between 23°C and 25°C and photoperiodic extension lighting was used over the winter months. Plants were watered by hand once per day.

2.4 Stereomicroscope and light microscopy
A Leica (MZFLIII) stereomicroscope equipped with a DC200 digital camera was used in the course of this research. It is referred to in the following text as the stereomicroscope. The light microscope used in the course of this research was an Axioplan microscope equipped with an Axiophot camera (Zeiss, Jena, Germany).

2.5 Scanning electron microscopy
2.5.1 Preparation of specimens
For scanning electron microscopy (SEM) of mature tissue, entire leaves and inflorescences were harvested in the field, placed in water and taken to the lab where small samples (less than 4 mm x 4 mm) were excised with a razor blade and fixed. Mature leaf tissue was fixed in 3% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at room temperature.

For analysis of younger tissues, seedlings were grown in the glasshouse and entire seedlings were taken to the lab, dissected under the stereomicroscope and fixed. Meristematic tissue was fixed in 3% glutaraldehyde and 2%
paraformaldehyde in 0.05 M phosphate buffer (pH 7.2) overnight at room
temperature.

Samples for SEM were dehydrated in a graded ethanol series, critical-point
dried in liquid CO₂ (Polaron E 3000 critical point dryer) and sputter coated with
25 nm gold (SCD-050 sputter coater; Bal-Tec, Balzers, Liechtenstein).

2.5.2 Preparation of replicas
Resin replicas of epidermal surfaces and developing prophylls were made using
a dental impression media method (Williams et al., 1987). Leaf tissue was
flattened onto double-sided tape on a glass microscope slide. Developing
axillary buds were dissected on glass microscope slides under the dissecting
microscope. Fresh dental impression media (Coltene® President light body
dental impression media, NJ, USA) was mixed on a parafilm strip and the
specimen was immediately pressed into it to create a mould. For more 3-
dimensional specimens, such as axillary buds, segments of plastic drinking
straw were cut to make small cylindrical containers. Dental impression media
was transferred to the straw segment immediately after mixing, and the
specimen was pressed into the centre. The media was allowed to set for five
minutes or longer.

Tissue was gently removed from the mould once the media was set. Casts were
then made by filling the mould with resin (Araldite super strength liquid epoxy
resin, Selleys) and applying a vacuum to remove air bubbles. These were left to
set overnight then replicas were gently removed and mounted on SEM stubs.
Replicas were sputter coated as described above.

2.5.3 Viewing and photography
Specimens were examined on a Cambridge 250 Mark III scanning electron
microscope (Cambridge Instruments, Cambridge, UK) operated at 20 kV, and
images were captured on 35 mm film.
2.6 Histology

2.6.1 Paraffin sections

Fixation
Tissue for paraffin embedding was fixed in FAA (3.5% formaldehyde, 5% acetic acid, 50% ethanol (v/v)). Excess tissue was removed under the stereomicroscope, and samples were transferred to vials containing FAA. These were then put into a desiccator and a vacuum was applied. Care was taken not to allow the fixative to boil. Samples were held under vacuum for 2-5 min, and then the vacuum was released slowly. FAA was replaced and the vials were left at room temperature for 2-3 h.

Dehydration
Dehydration steps were carried out at 4°C. Fix was removed by pouring or pipetting and samples were rinsed with 50% ethanol (v/v). Samples were run through a graded ethanol series (50%, 70%, 85%, 95% + 0.1% eosin, 100%, 100% for 90 min each), then left in 100% ethanol overnight.

Histoclear infiltration
Histoclear (National Diagnostics) infiltration was carried out at room temperature. Samples were treated with 100% ethanol for 2 h, 50% histoclear: 50% ethanol for 1 h and three changes of 100% histoclear for 1 h each.

Paraffin infiltration
Paraplast chips (Mc Cormick Scientific) were added to approximately half the volume of histoclear. Vials were transferred to a 55°C oven and left overnight. The following morning, the molten paraffin and histoclear solution was poured off. Molten paraplast was then replaced twice per day for three days or three times per day for two days.

Casting blocks
Paraffin blocks were cast in foil candy cups (Home Style Chocolates). Foil cups were placed on a 55°C hot plate and filled with fresh molten paraffin. Tissue samples were transferred to candy cups using clean forceps and positioned in the correct orientation. Cups were then transferred to a cool surface such as an
inverted petri dish in an ice box to set. Paraffin blocks were stored at 4°C until required.

**Sectioning paraffin embedded material**

5-10 μm sections were cut on a microtome (Leica model Jung RM 2045). Wax ribbons were transferred to a 42°C water bath (Leica model HI 1210) to relax any creases in the ribbon. Ribbons were floated onto microscope slides (ProbeOn Plus, Fisher Scientific) and positioned using toothpicks. Slides were held vertically to drain, and excess water was removed by blotting with a Kim wipe (Kimberly-Clark). Slides were then placed on a 42°C hot plate (Leica model HI 1220) and allowed to dry overnight.

**2.6.2 Staining**

**Toluidine Blue**

The protocol used for toluidine blue staining of paraffin sections is adapted from Ruzin (1999). Paraffin sections were stained in 0.05% (w/v) toluidine in water for 30 min before removal of the paraffin. Slides were rinsed in water and air dried. Paraffin was removed with histoclear (first rinse 10 min, second rinse 5 min). Coverslips were mounted with DPX (dibutylphthalate (10 ml) + polystyrene (25 g) + xylene (70 ml)) (Ruzin, 1999).

**Safranin and fast green**

The protocol for safranin and fast green staining of paraffin sections is adapted from Johansen (1940). Slides were placed in metal slide racks, and all steps were carried out at room temperature. Slides were dewaxed by immersing in histoclear for 10 min and then in fresh histoclear for a further 10 min. Slides were run through a graded ethanol series and then stained in safranin solution (1% (w/v) safranin in 2:1:1 methyl cellosolve : 95% ethanol : water) for 24 - 48 h. Slides were rinsed in distilled water until the water ran clean, immersed in 70% ethanol for 5 min and then destained in picric acid (0.5% (w/v) picric acid in 95% ethanol) for 10 s. Slides were rinsed in 95% ethanol for 1 min, then in a second solution of 95% ethanol for 10 s, stained in fast green solution (0.15% (w/v) fast green in 1:1:1 methyl cellosolve : ethanol : clove oil) for 10 – 15 s, then immediately rinsed in clove oil. Slides were then treated with the following
solutions; clove oil: histoclear for 10 s, histoclear: ethanol for 5 min, and two changes of histoclear for 5 min each. Coverslips were mounted with DPX.

2.6.3 Resin sections
Leaf tissue was fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at room temperature. Samples were infiltrated and embedded in Procure 812 (ProSciTech, Kelso, Australia). Sections (1µm) were cut, heat mounted and stained with 0.05% toluidine blue in water and photographed under the light microscope.

2.6.4 Distinguishing xylem and phloem in sectioned material
The vessel elements of the xylem have lignified cell wall thickenings and lack protoplasts. Helical thickenings of vessel elements can be identified by examining serial sections. In tissue that has been stained with toluidine blue, lignified cell wall thickenings stain a lighter colour than other cell walls. In tissue stained with safranin and fast green, the lignified cell walls of the vessel elements stain bright red (Ruzin, 1999). Cytoplasm and non-lignified cell walls stain green. The phloem is comprised of smaller, non-lignified sieve tube elements with companion cells.
3. **Wavy auricle in blade1-R**

3.1 **Introduction**

*Wab1-R* is a dominant mutation characterised by ectopic auricle and sheath-like tissue in the leaf blade. *Wab1-R* leaf blades are narrow and have fewer lateral veins than wild-type leaves (Hay and Hake, 2004). The *Wab1-R* allele used in the current study arose in androgenous tissue culture and was recovered by James Wassom (Hake *et al*., 1999).

Initial characterisation of the *Wab1-R* phenotype was undertaken by Angela Hay as part of a PhD project supervised by Sarah Hake (Plant Gene Expression Center, Albany, CA). This work has subsequently been published in *Plant Physiology* (Hay and Hake, 2004). A second publication in *Development* details a mosaic analysis of *Wab1-R* and an analysis of *lg1-R* leaf shape undertaken by Toshi Foster, Sarah Hake and myself (Foster *et al*., 2004). *Lg1* expression data provided by Angela Hay is also included in the *Development* publication.

The liguleless mutants, *lg1* and *lg2*, delete ligule and auricle tissues (Emerson, 1912; Brink, 1933). Genetic analysis indicates that these genes act in a common pathway to specify ligule and auricle development (Harper and Freeling, 1996). *Lg1* is expressed at low levels in the ligular region of developing leaf primordia and acts cell-autonomously to specify ligule and auricle tissues, whereas, *Lg2* acts non-autonomously (Becraft *et al*., 1990; Harper and Freeling, 1996; Moreno *et al*., 1997). A mosaic analysis of *lg1-R* found that the ligule and auricle reinitiate in a more basal position on the marginal side of *lg1-R/-* sectors (Becraft and Freeling, 1991). This analysis supports a model in which *Lg1* function is required for the transmission of an inductive signal.

Crossing *Wab1-R* to *lg1-R* mutants revealed a novel phenotype. Unlike *lg1-R* mutants, which have distinct regions of blade and sheath, the blade-sheath boundary of *lg1-R;Wab1-R* double mutants is severely disrupted, with strips of sheath-like tissue extending along the length of the blade (Hay and Hake, 2004). The leaf blade of *lg1-R;Wab1-R* double mutants is much narrower than
either of the single mutants. *Lg1* is expressed earlier and more distally in *Wab1-R* mutant leaf primordia, further suggesting an interaction between the two genes (Foster et al., 2004). To further elucidate interactions between *Lg1* and *Wab1-R*, mosaic analyses of *Wab1-R* in *Lg1* and *lg1-R* backgrounds and of *lg1-R* in a *Wab1-R* background were conducted. To better understand the nature of the *Wab1-R* narrow leaf phenotype, an analysis of vascular development and a cell lineage analysis in *Wab1-R* leaves were conducted.

### 3.2 Specific materials and methods

#### 3.2.1 Phenotypic analysis of *Wab1-R* and *lg1-R;Wab1-R* plants

F3 families segregating 1:1 for *lg1-R* : *lg1-R;Wab1-R*/*wab1* were used for phenotypic analyses. Tissue samples from specific regions of the blade and sheath (see Figure 3.4) were fixed for SEM and light microscopy.

#### 3.2.2 Mosaic and clonal analyses

**Genetic stocks**

Crosses for the mosaic and clonal analyses were carried out by Sarah Hake. Introgressed material was provided by Sarah Hake and Angela Hay.

*Mosaic analysis of *Wab1-R* and clonal analysis of *lg1-R* leaves*

For the mosaic analysis of *Wab1-R*, heterozygous *Wab1-R*/*wab1* plants were crossed to Maize Genetics Coop stocks heterozygous for *white seedling3-R* (*w3-R*). For the mosaic analysis of *Wab1-R* in a *lg1-R* background, *lg1-R/lg1-R;Wab1-R*/*wab1* plants were crossed to *lg1-R/lg1-R; v4-R w3-R/v4-R W3* stocks from the Maize Genetics Coop (Figure 3.1).

The same stock (*lg1-R* and *Lg1* plants) was also used for the clonal analysis of *lg1-R* leaves, as the *w3-R* marker gene is on the opposite chromosome arm to *lg1* and is therefore considered to be unlinked to *lg1*.
Radiation-induced chromosome breakage, followed by mitosis.

Figure 3.1. Scheme for mosaic analysis of Wab1-R. (A) Seeds are heterozygous for W3 and Wab1-R in coupling, in a Lg1 background. w3-R is an albino marker gene. Irradiating seeds causes chromosome breakages. When the chromosome arm carrying W3 and Wab1-R is lost, the recessive w3-R and wab1 alleles are uncovered in that cell. Cells that are clonally derived from this cell are albino and carry only the non-mutant wab1 allele. Control plants are heterozygous for W3 and homozygous for the wild-type wab1 allele. (B) Seeds are heterozygous for W3, V4 and Wab1-R in coupling, or heterozygous for V4 and Wab1-R and homozygous for W3, in a lg1-R background. Loss of the chromosome arm carrying W3 and Wab1-R results in white, wab1/- sectors. Loss of V4 and Wab1-R results in yellow wab1/- sectors.
Mosaic analysis of Ig1-R in Wab1-R background

For the mosaic analysis of Ig1-R in Wab1-R and wab1 backgrounds, heterozygous Wab1-R plants were cross to Maize Genetics Coop stocks heterozygous for Ig1-R and the linked marker gene albescent-R (al-R) (Figure 3.2).

Clonal analysis of Wab1-R leaves

For the clonal analysis of Wab1-R leaves, heterozygous Wab1-R/wab1 plants were cross to Maize Genetics Coop stocks heterozygous for the unlinked marker gene lemon white-R (lw-R).
Irradiation conditions
Seeds were imbibed for 48 hours at 25°C then irradiated with approximately 1,500 rads. The irradiation utilised a 6 MV photon (X-ray) beam generated by a linear accelerator at the Palmerston North Hospital Radiotherapy Unit, Palmerston North, New Zealand.

Sector analysis
Plants were grown to maturity and screened for albino (w3-R/- or lw-R/- or al-R/-), and yellow (v4-R/-) sectors throughout development. All sectored leaves were harvested at maturity and photographed and/or photocopied. Leaf number, sector width and the lateral position of the sector within the blade and sheath were recorded.

Transverse hand sections of freshly harvested sectored leaves were examined by epifluorescence microscopy using a Leica (MZFLII) stereomicroscope equipped with a 395-440 nm excitation filter and a 470 nm observation filter. All sections were photographed using a DC200 digital camera (Wetzlar, Germany). Under these conditions, normal chloroplasts fluoresce bright red and cell walls appear blue-green. No chlorophyll autofluorescence is detected in w3-R/- or al-R/- cells. The presence or absence of chlorophyll in epidermal layers was scored by inspecting guard cells, the only chloroplast-containing cell-type in the epidermis.

Sectors of v4-R/- appear yellow due to a delay in the accumulation of chlorophyll, but eventually become green. Sector boundaries of v4-R/- sectors were marked with a pen. Samples of leaf tissue that spanned sector boundaries were fixed for SEM. Prior to fixation, a small notch was made at the sector boundary.

Measurements of mosaic leaves
For the mosaic analyses, the sectored and non-sectored sides of each leaf were measured from midrib to margin at three points along the leaf: blade midpoint, blade-sheath boundary, and sheath midpoint. The width of the non-sectored leaf-half was subtracted from that of the sectored half to give an absolute difference in leaf-half width. For each genotype and measurement position, data were analysed using a non-parametric 1-sign test to determine if
the median values were significantly different than 0. This test was used because data from Wab1-R and Ig1-R;Wab1-R leaves are not normally distributed. For the mosaic analysis of Wab1-R, the lateral position of each sector was represented as the distance from the midrib to the closest sector border, divided by the width of the entire leaf-half (see Table 3.5). Statistical analysis was performed using Origin 6.0 (Microcal Software, Inc., Northampton, MA, USA) and MINITAB (State College, PA, USA).

Clonal analysis of Ig1-R leaves
For the comparison of clonal, w3-R-marked sectors in wild-type and Ig1-R plants, sector widths were measured at the blade-sheath boundary. To minimise variation in leaf shape, only leaves 9-17 were included in this analysis. The median sector width was calculated separately for sectors near the midrib (lateral position 0-0.29) and in lateral plus margin domains (0.3-1.0). Data from wild-type and Ig1-R plants were compared using a Kruskal-Wallis test. This test was used because the data from Ig1-R plants are not normally distributed.

Clonal analysis of Wab1-R leaves
To investigate the nature of the Wab1-R narrow leaf phenotype, a clonal analysis was undertaken. Half-leaf width (from midrib to margin) and sector width were measured at the blade midpoint, at the base of the blade, and in the culm. The mean sector width was calculated separately for sectors near the midrib (lateral position 0-0.29), in the lateral domain (0.3-0.59), and near the margin (0.6-1.0). Means were compared by Student’s t-test to determine if they were significantly different at the 0.05 confidence level. Data were analysed using Microsoft Office Excel 2003.

The position of a sector within the culm was used to approximate the radial position of the sector within the SAM from which that phytomer arose. This method is similar to that employed by Scanlon (2000) in the mosaic analysis of ns. The radial point of the culm corresponding to the midrib of the leaf above was designated as 0°. Measurements were made from 0° to the inner boundary of the sector, from 0° to the outer sector boundary, and of the entire culm circumference. The radial position of each sector in the culm was calculated in degrees from the midrib (see Figure 3.8) using the following equations:
Position of inner sector boundary = \(\frac{S_i}{C} \times 360\)

Position of outer sector boundary = \(\frac{S_o}{C} \times 360\)

Where:

\(S_i\) = distance from midrib to inner sector boundary

\(C\) = circumference of culm

\(S_o\) = distance from midrib to outer sector boundary

Thus, for a sector that begins 8 mm from the midrib, in a culm with a circumference of 60 mm, the position of the inner sector boundary is calculated as \(\frac{8}{60} \times 360 = 48^\circ\).

### 3.2.3 \(lg1-R\) leaf measurements

The \(lg1-R\) mutation was introgressed at least four times into the W23 inbred background and plants were grown to maturity in the Gill Tract nursery, Albany, CA. The 9th, 10th, and 11th leaves down from the tassel were measured from \(lg1-R\) and \(Lg1/lg1-R\) siblings. Leaf width was measured at the blade-sheath boundary, and blade and sheath length were measured along the midrib. Data were compared by one-way ANOVA.

### 3.2.4 Lateral vein count in \(Wab1-R\) leaf primordia

\(Wab1-R\) leaves have fewer lateral veins than wild-type leaves (Hay and Hake, 2004). To determine when this defect is first apparent, lateral veins were counted in early stage leaf primordia. This analysis was done in B73 stock and \(Wab1-R\) introgressed seven times into a B73 background. \(Wab1-R\) homozygotes were used, as the phenotype is more severe in homozygotes than in heterozygotes (Hay and Hake, 2004). Seedlings were harvested at 12 to 16 d after germination. Outer leaves were removed and the apices were fixed in FAA and paraffin embedded. 10 μm transverse sections were cut, mounted on slides and stained with toluidine blue. Leaf primordia 9-12 were examined. Developing lateral veins were counted in sections 20μm above the base of the primordium. Developing procambial strands were identified by cell divisions at angles that disrupt the parallel arrangement of cells (Sharman, 1942; Esau, 1960).
The mean number of lateral veins was calculated separately for Wab1-R and wild-type leaf primordia at each plastochron stage (P1- P6). The means for each plastochron were compared by Student’s t-test to determine if they were significantly different at the 0.05 confidence level. Data were analysed using Microsoft Office Excel 2003.

3.3 Results

3.3.1 Ig1-R enhances the Wab1-R mutant phenotype

Four distinct tissue types demarcate the proximal-distal axis of the maize leaf, the proximal sheath and distal blade are separated by ligule and auricle tissues (Figure 3.3 A, E). The ligule is an epidermally-derived fringe, and the auricles are thickened wedges of tissue that act as a hinge between blade and sheath (Sharman, 1941; Becraft et al., 1990). Each of these tissue types has characteristic epidermal features and histological organisation, which have been well characterised by scanning electron and light microscopy (Sharman, 1942; Esau, 1977; Russell and Evert, 1985; Langdale et al., 1989; Sylvester et al., 1990).

The Wab1-R mutation disrupts normal patterning of the leaf and results in patches of ectopic auricle, sheath and ligule in the leaf blade (Figure 3.3 F; Hay and Hake, 2004). Often a localised increase in blade width occurs immediately distal to patches of ectopic auricle. In addition, the normally placed auricle is more extensive, spreading distally into the leaf blade. Long strips of thickened auricle tissue and the reduced lamina width give Wab1-R plants an unusual appearance; narrow, rigid leaves extend from the main axis at a more obtuse angle than wild-type leaves (Figure 3.3 B). Examination of histological and epidermal features reveals that the Wab1-R blade contains cells with auricle and sheath identity (Figure 3.4 I, J, L). In both ectopic sheath and auricle tissue, intermediate veins fuse into lateral veins, and normal bundle sheath anatomy is absent or incomplete (arrowheads in Figure 3.4 I, J).

We constructed double mutants between Wab1-R and Ig1-R to analyse the effect that loss of auricle tissue would have on the Wab1-R phenotype. Recessive Ig1 mutations remove ligule and auricle, giving the mutant leaves a
more upright appearance (Figure 3.3 D) (Emerson, 1912). Despite the lack of ligule and auricle, \( lg1-R \) leaves have separate blade and sheath domains (Figure 3.3 H). \( lg1-R;Wab1-R \) double mutants exhibit a striking, narrow leaf phenotype (Figure 3.3 C, G). Both the normal and ectopic auricle tissue is absent in the double mutant, most of the proximal blade is deleted, and sheath-like tissue extends along the margins of the residual blade.

![Figure 3.3. Leaf and whole plant phenotypes.](A) Wild-type, (B) Wab1-R, (C) \( lg1-R;Wab1-R \), and (D) \( lg1-R \) plants 8 weeks after planting. Adaxial view of blade-sheath boundary of (E) wild-type, (F) Wab1-R, (G) \( lg1-R;Wab1-R \), and (H) \( lg1-R \) leaves. b=blade, s=sheath, lg=ligule, a=auricle, ea=ectopic auricle, ae=auricle extension, es=ectopic sheath. Arrowhead in (G) indicates presumptive blade-sheath boundary in \( lg1-R;Wab1-R \) leaf.

The ectopic tissue in \( lg1-R;Wab1-R \) leaf blades has histological and epidermal features similar to sheath tissue. The adaxial surface is hairless and cells are long with smooth cell wall junctions (Figure 3.4 M), while the abaxial surface is covered with hairs specific to abaxial sheath tissue (not shown). \( lg1-R;Wab1-R \) sheath-like tissue is very thin in the transverse dimension (Figure 3.4 K) and the intervascular spacing and prominent transverse veins resemble those normally found in marginal sheath tissue (Figure 3.4 G). In distal positions and near the midrib, \( lg1-R;Wab1-R \) leaves have normal blade tissue (not shown). Sheath tissue identity is not affected by the \( lg1-R \) or \( Wab1-R \) mutations.
Figure 3.4. Epidermal and histological features of wild-type, Wab1-R and Ig1-R;Wab1-R leaves. SEM of adaxial surface of wild-type (A) blade, (B) auricle and (C) sheath. Transverse section through wild-type (D) blade, (E) auricle, (F) internal sheath and (G) marginal sheath tissue. (H) Cartoon depicting regions where tissue was sampled. Transverse sections through (I) ectopic auricle in Wab1-R blade, (J) ectopic sheath in Wab1-R blade and (K) ectopic sheath in Ig1-R,Wab1-R blade. SEM of adaxial surface of (L) Wab1-R ectopic auricle and (M) Ig1-R;Wab1-R ectopic sheath. All sections are oriented with the adaxial surface upwards. Arrows in (A) and (O) indicate multicellular base of macrohair. Normal bundle-sheath anatomy indicated by arrowhead in (D), abnormal bundle-sheath anatomy indicated by arrowheads in (I) and (J). Scale bars = 100 μm.

3.3.2 Ig1-R alters leaf shape

Although the Ig1 ligule defect has been well described by others, the altered shape of Ig1 leaves has not been reported. We found that Ig1-R leaves are significantly narrower at the blade-sheath boundary than Lg1/Lg1-R siblings (Table 3.1). The mean width of the ninth leaf counting down from the tassel was 76 mm for Ig1-R plants, whereas, the mean width was 102 mm for wild-type siblings. A similar trend was seen for the tenth and eleventh leaves down from the tassel. We also noted that while the overall lengths of Ig1-R and Lg1/Lg1-R leaves are the same, Ig1-R blades are shorter and Ig1-R sheaths are longer than those of Lg1/Lg1-R siblings (Table 3.1). This finding indicates that the blade-sheath boundary is established in a more distal position in the Ig1-R mutant.
Table 3.1. Comparison of *lg1-R* and wild-type (*Lg1/lg1-R*) leaf shape. Sheath length, blade length, and leaf width at the blade-sheath boundary were measured for *lg1-R/lg1-R* and *Lg1/lg1-R* siblings. Data were compared by one-way ANOVA. Means of all measurements are in mm. Leaves were numbered counting down from the tassel. Sample sizes are as follows; *lg1-R/lg1-R* (N=21), *Lg1/lg1-R* (N=13). * indicates values that are significantly different at the 0.05 confidence level. SE = standard error. The schematic shows measurement positions and illustrates *lg1-R/lg1-R* and *Lg1/lg1-R* leaf shape. W = width.

A

<table>
<thead>
<tr>
<th>Ninth leaf down</th>
<th><em>Lg1/lg1-R</em></th>
<th><em>lg1-R/lg1-R</em></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leaf length</td>
<td>Mean 903</td>
<td>Mean 893</td>
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</tr>
<tr>
<td></td>
<td>SE 17.4</td>
<td>SE 10.5</td>
<td></td>
</tr>
<tr>
<td>Blade length</td>
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<td>Mean 649</td>
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<tr>
<td></td>
<td>SE 15.2</td>
<td>SE 8.6</td>
<td></td>
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<tr>
<td>Sheath length</td>
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<td>Mean 243</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>SE 3.7</td>
<td>SE 3.7</td>
<td></td>
</tr>
<tr>
<td>Blade width</td>
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<td>Mean 76</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>SE 2.9</td>
<td>SE 2.2</td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
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<tr>
<th>Tenth leaf down</th>
<th><em>Lg1/lg1-R</em></th>
<th><em>lg1-R/lg1-R</em></th>
<th>P-value</th>
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</thead>
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<tr>
<td>Total leaf length</td>
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<td>Mean 846</td>
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<td>SE 33.3</td>
<td>SE 17.7</td>
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<tr>
<td>Blade length</td>
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<td>Mean 605</td>
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<tr>
<td></td>
<td>SE 21.0</td>
<td>SE 13.6</td>
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<tr>
<td>Sheath length</td>
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<td>Mean 243</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>SE 4.3</td>
<td>SE 4.0</td>
<td></td>
</tr>
<tr>
<td>Blade width</td>
<td>Mean 92</td>
<td>Mean 65</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>SE 2.3</td>
<td>SE 2.3</td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Eleventh leaf down</th>
<th><em>Lg1/lg1-R</em></th>
<th><em>lg1-R/lg1-R</em></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leaf length</td>
<td>Mean 789</td>
<td>Mean 762</td>
<td>0.537</td>
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<tr>
<td></td>
<td>SE 38.7</td>
<td>SE 22.6</td>
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<tr>
<td>Blade length</td>
<td>Mean 575</td>
<td>Mean 521</td>
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</tr>
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<td></td>
<td>SE 35.4</td>
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<td></td>
</tr>
<tr>
<td>Sheath length</td>
<td>Mean 212</td>
<td>Mean 242</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>SE 3.9</td>
<td>SE 3.1</td>
<td></td>
</tr>
<tr>
<td>Blade width</td>
<td>Mean 76</td>
<td>Mean 54</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>SE 2.5</td>
<td>SE 2.1</td>
<td></td>
</tr>
</tbody>
</table>
We compared the width of w3-R-marked clonal sectors in wild-type (Lg1/Ig1-R) and lg1-R plants. Sectors were measured at the base of the blade. Sectors located near the midrib had similar median widths in wild-type and lg1-R blades. However, sectors in lateral and marginal regions were significantly narrower in lg1-R mutants than in wild-type leaves (Table 3.2). These data indicate that the lg1-R lateral growth defect is localised to lateral and marginal domains at the base of the blade.

Table 3.2. Median width of clonal sectors in Lg1/Ig1-R and Ig1-R/Ig1-R plants, measured at the blade-sheath boundary. Median sector widths were calculated separately for sectors near the midrib (0-0.29) and sectors in lateral and marginal positions (0.3-1.0). Data were compared by the Kruskal-Wallis test for non-parametric distribution of data. * indicates values that are significantly different at the 0.05 confidence level.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Lateral position of sector</th>
<th>Median sector width (mm)</th>
<th>P-value</th>
</tr>
</thead>
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<td>0-0.29</td>
<td>1.0</td>
<td>0.453</td>
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<td>lg1-R/Ig1-R</td>
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<td>0-0.29</td>
<td>1.0</td>
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<tr>
<td>Lg1/Ig1-R</td>
<td>18</td>
<td>0.3-1.0</td>
<td>5.0</td>
<td>0.007*</td>
</tr>
<tr>
<td>lg1-R/Ig1-R</td>
<td>45</td>
<td>0.3-1.0</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3 Mosaic analysis of Wab1-R

Sectors of tissue lacking the dominant Wab1-R allele (wab1/-) were created in both Wab1-R and lg1-R;Wab1-R mutants to determine if Wab1-R disrupts leaf patterning in a cell-autonomous manner (Figure 3.1). Stocks carrying Wab1-R in repulsion to w3-R were X-irradiated to induce random chromosome breaks. Radiation-induced breaks proximal to W3 resulted in albino, non-Wab1 (w3-R wab1/-) sectors in otherwise green, Wab1-R or lg1-R;Wab1-R plants. In lg1-R;Wab1-R plants, chromosome breaks proximal to V4 created yellow, lg1-R (v4-R wab1/-;lg1-R/lg1-R) sectors. The loss of W3 in normal plants, and either W3 or V4 in lg1-R plants, provided control sectors that were hemizygous for chromosome 2L.

Out of 1681 irradiated seeds, 93 w3-R wab1/- sectored leaves were identified in 42 Wab1-R/wab1 plants and 51 sectored leaves were identified in 32
wab1/wab1 control plants. In the second experiment, 4,608 seeds were irradiated; 115 w3-R wab1/- sectors and 65 v4-R wab1/- sectors were identified in 81 lg1-R/lg1-R; Wab1-R/lwab1 plants. In 46 lg1-R/lg1-R; wab1/wab1 control plants, 90 w3-R wab1/- and 50 v4-R wab1/- sectors were analysed.

To ensure that the chromosome arm carrying Wab1-R was lost early in leaf development, only sectors that extended through both the sheath and blade were analysed. Given the variability of the Wab1-R phenotype, only sectors adjacent to tissue displaying a mutant phenotype could be scored. Thus, of 273 total sectors, only 77 were scorable for tissue identity. Sectors adjacent to ectopic auricle and sheath tissue were analysed for phenotypic expression (mutant or wild-type) and cell layer composition (green or albino) (Table 3.3). Because v4-R sectors eventually accumulate normal amounts of chlorophyll, it was difficult to determine the internal layer composition of yellow, v4-R sectors in mature leaves. Thus, only w3-R sectors were scored for albino versus green mesophyll and epidermal layers. We predicted that white or yellow (wab1/-) sectors would have normal blade tissue if Wab1-R functions cell autonomously, whereas the sectors would have the same mutant phenotype as the adjacent green tissue if Wab1-R acts in a non-autonomous manner.

**Ectopic auricle and auricle extension phenotypes in Wab1-R mutants**

Sectors were examined using SEM and handsections to determine the phenotype of wab1/- tissue. In 77% (24/31) of scorable sectors, wab1/- cells exhibited normal blade characteristics whereas adjacent Wab1-R tissue displayed either ectopic auricle or extension of auricle phenotypes (Table 3.3, Figure 3.5 A-F). These results indicate that Wab1-R generally acts in a cell-autonomous manner in the lateral dimension to condition ectopic auricle and auricle extension phenotypes.

Figure 3.5 F is a SEM of the adaxial epidermis of the boxed region shown in Figure 3.5 C. There is a clear transition from albino wab1/- tissue, which has blade characteristics such as macrohairs (left of arrowhead), to green, hairless Wab1-R/wab1 tissue with unexpanded dovetailed cells typical of immature auricle (right of arrowhead, Figure 3.5 F). Green, Wab1-R/wab1 tissue fluoresces red under UV illumination while albino wab1/- tissue appears blue-
green. When sectored tissue is viewed in transverse section, abrupt changes in histology are apparent at the sector boundaries. For example, in Figure 3.5 G, the albino \textit{wab1/-} tissue has characteristics of blade tissue, whereas the adjacent \textit{Wab1-R/wab1} tissue is thicker and auricle-like. The SEM in Figure 3.5 H shows the same sector boundary, with albino tissue to the left of the arrowhead and green tissue to the right. The green \textit{Wab1-R/wab1} tissue has larger mesophyll cells and is densely covered by long hairs without multicellular bases; these cells are characteristics of mature auricle tissue. The albino \textit{wab1/-} tissue has prickles, macrohairs and cell types typical of blade tissue.

Of the seven sectors that displayed auricle characteristics through all or part of the sector, six had one or more inner layers of green, \textit{Wab1-R} cells (Table 3.3). These results indicate that \textit{Wab1-R} generally acts cell-autonomously in the lateral dimension, but may act non-cell autonomously between cell layers.

Table 3.3. Summary of \textit{wab1/-} sector phenotypes. Sectors were scored as “+” if they displayed characteristics of blade tissue, and “W” if they exhibited auricle or sheath characteristics. Mixed layer sectors that spanned fewer than three veins were included as part of the larger adjacent sector. If adjacent sector types displayed different phenotypes then both were scored. Otherwise, only the sector composition adjacent to green, \textit{Wab1-R} tissue was scored. *Four yellow (\textit{v4-R}) sectors were not included in sector subtypes due to difficulties in determining the exact layer composition of \textit{wab1 v4-R/-} sectors.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Scorable sectors</th>
<th>All sector types</th>
<th>white L1/white L2</th>
<th>green L1/white L2</th>
<th>white L1/mixed L2</th>
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<tr>
<td>\textit{Wab1}</td>
<td></td>
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<tr>
<td>Ectopic auricle</td>
<td>31</td>
<td>24 + (77%)</td>
<td>4</td>
<td>17</td>
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<td>Auricle extension</td>
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<td>2</td>
<td>0</td>
<td>4</td>
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<td>\textit{Wab1}</td>
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<td></td>
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<tr>
<td>Ectopic sheath</td>
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<td>15 + (75%)</td>
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<td>14</td>
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<td>1</td>
</tr>
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<td></td>
<td></td>
<td>5 W (25%)</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>\textit{Ig1-R;Wab1}</td>
<td>26*</td>
<td>26 + (100%)</td>
<td>10</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Ectopic sheath</td>
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<td>0 W (0%)</td>
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</tbody>
</table>
Figure 3.5. Phenotypes of wab1/- sectors in Wab1-R leaves. (A-E) Abaxial view of Wab1-R leaves exhibiting auricle extension and/or ectopic auricle phenotypes with albino wab1/- sectors exhibiting normal blade characteristics. (F) Scanning electron micrograph of adaxial surface of boxed region shown in (C), illustrating unexpanded, dovetailed auricle-like cells in Wab1-R/wab1 ectopic auricle tissue (right of arrowhead), and normal blade epidermal characteristics such as macrohairs in the wab1/- sector (left of arrowhead). (G) Fluorescence micrograph of a transverse section through a sector adjacent to auricle extension, illustrating a sharp boundary between green, Wab1-R/wab1 auricle-like tissue (fluoresces red), and albino, wab1/- tissue (appears blue-green). (H) SEM of adaxial surface of sector boundary shown in (G), exhibiting hairy, fully expanded auricle-like cells in Wab1-R/wab1 tissue (right of arrowhead) and normal blade cells including prickle hairs and macrohairs in wab1/- tissue (left of arrowhead). (I) Fluorescence micrograph of a transverse section through the sector shown in (J). Green, Wab1-R/wab1 tissue is very thin with widely spaced veins, a hairless adaxial surface and abaxial hairs characteristic of marginal sheath tissue. Albino wab1/- tissue has the histological organization of normal blade tissue. (J) Abaxial view of sector adjacent to ectopic sheath tissue, arrowhead marks sector boundary. (K) Cartoon depicting an albino wild-type (wab1/-) sector in a green Wab1-R/wab1 leaf. Scale bar = 500μm in F, and 100μm in G-I.
An interesting pattern was observed in Wab1-R plants with mild auricle extension phenotypes. In most cases, sectors in these plants had auricle extension to the midrib side of the sector, but recovered normal tissue identity both within the sector and on the marginal side of the sector (e.g. Figure 3.5 A, B). In plants exhibiting more severe phenotypes such as ectopic auricle and sheath, sectors with normal tissue identity were flanked by mutant tissue on both sides. These results suggest that normal (wab1) cells may have a directional effect on adjacent Wab1-R cells. There was no obvious relationship between recovery of tissue identity and sector size.

Ectopic sheath tissue in Wab1-R and Ig1-R;Wab1-R mutants

In Wab1-R mutants, 75% (15/20) of the sectors adjacent to ectopic sheath-like tissue exhibited normal blade characteristics (Table 3.3). These results also indicate that Wab1-R generally disrupts tissue patterning in a cell-autonomous manner. Figure 3.5 J shows an albino sector adjacent to a region of ectopic marginal sheath tissue, and Figure 3.5 I is a transverse section through this sector boundary. The green, Wab1-R/wab1 mutant tissue has characteristics of marginal sheath tissue; it is thin, has widely spaced veins, the adaxial surface is hairless, and the abaxial surface has long hairs without multicellular bases (Figure 3.5 I). In contrast, the adjacent albino wab1/- tissue exhibits histological organisation and epidermal features specific to normal blade tissue (Figure 3.4 D).

In Ig1-R;Wab1-R double mutants, all (26/26) scorable wab1/- sectors exhibited normal blade characteristics, indicating that Wab1-R acts completely autonomously in the absence of Lg1 (Table 3.3). The widest sectors were located at the margin, and restored the leaf half to a more normal shape and width (Figure 3.6 A, B). Figure 3.6 A shows a yellow wab1 v4-R/- sector that occurred at the margin. The yellow blade tissue has almost doubled the width of the leaf base. The sector shown in Figure 3.6 C was sectioned and examined by SEM (Figure 3.6 D, E). In transverse section, there is a sharp boundary between albino wab1/- blade tissue and green Wab1-R/wab1 tissue with veins appressed against the abaxial surface, typical of sheath (Figure 3.6 D). The SEM shows crenulated blade cells to the left of the sector boundary (arrowhead), and smooth-walled, elongated sheath-like cells to the right (Figure
3.6 E). Figure 3.6 F is a transverse section through another sector boundary, illustrating the abrupt transition between albino blade tissue and green tissue with long abaxial hairs and other characteristics typical of marginal sheath.

Figure 3.6. Phenotypes of wab1/- sectors in Ig1-R;Wab1-R leaves. (A) Abaxial view of yellow v4-R wab1/- sector, and (B) adaxial and (C) abaxial view of white, w3-R wab1/- sectors adjacent to ectopic sheath tissue. Arrowheads in (A) and (B) mark sector borders. (D) Fluorescence micrograph of a transverse section through the inner sector boundary of leaf shown in (C). Green Ig1-R;Wab1-R tissue has histological organisation of sheath, and albino wab1/- tissue exhibits blade histology. (E) SEM of adaxial surface of sector boundary shown in (C) and (D), illustrating the sharp boundary between epidermal cell types in Ig1-R;Wab1-R/wab1 tissue (right of arrowhead) and Ig1-R;wab1/- tissue (left of arrowhead). (F) Fluorescence micrograph of a transverse section through another sector adjacent to marginal sheath-like tissue. The adaxial surface of green sheath-like tissue in (D) and (F) is hairless, whereas the albino tissue has hairs specific to blade. Scale bar = 100μm in (D-F).

In summary, mixed layer sectors behaved differently in Wab1-R and Ig1-R;Wab1-R plants. In Wab1-R plants, some mixed layer sectors exhibited the Wab1-R phenotype, whereas none of the mixed layer sectors in Ig1-R;Wab1-R plants exhibited the Wab1-R phenotype. Thus, Wab1-R may act non-autonomously between layers or laterally, but only in a Lg1 background.

Effect of wab1/- sectors on leaf width
The leaf blades of Wab1-R and especially Ig1-R;Wab1-R mutants are significantly narrower than those of wild-type siblings (Hay and Hake, 2004). To investigate the effect of wab1/- sectors on Wab1-R and Ig1-R;Wab1-R leaf width, the width of sectored and non-sectored halves of each leaf were measured and compared. In both Wab1-R and Ig1-R;Wab1-R plants, there is a small but significant increase in the median width of the sectored half of the blade relative to the non-sectored half (Table 3.4). Measurements made at the
sheath midpoint show no significant difference in width between leaf-halves, indicating that *Wab1-R* specifically disrupts lateral growth of blade tissue (Table 3.4). No difference between the widths of sectored and non-sectored leaf-halves was found in wild-type and *lg1-R* control plants.

Table 3.4. Median differences in the width of wab1/- sectored and non-sectored leaf-halves at the blade-sheath boundary and sheath midpoint. The sectored and non-sectored sides of each leaf were measured from midrib to margin at the blade-sheath boundary and sheath midpoint. The width of the non-sectored leaf-half was subtracted from that of the sectored half to give an absolute difference in leaf-half widths. A value greater than 0 indicates that the sectored half of the leaf is wider than the non-sectored half. The data were analysed using a non-parametric 1-sign test to determine if the median values were significantly different from 0. * indicates values that are significantly different from 0 at the 0.05 confidence level.

<table>
<thead>
<tr>
<th>Position of measurement</th>
<th>Genotype</th>
<th>N</th>
<th>Median difference (mm)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blade-sheath</td>
<td>Wild-type</td>
<td>51</td>
<td>0.0</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Wab1-R</td>
<td>93</td>
<td>1.0</td>
<td>0.013*</td>
</tr>
<tr>
<td></td>
<td><em>lg1-R</em></td>
<td>118</td>
<td>0.0</td>
<td>0.146</td>
</tr>
<tr>
<td></td>
<td><em>lg1-R; Wab1-R</em></td>
<td>164</td>
<td>0.5</td>
<td>0.001*</td>
</tr>
<tr>
<td>Mid-sheath</td>
<td>Wild-type</td>
<td>29</td>
<td>0.0</td>
<td>0.189</td>
</tr>
<tr>
<td></td>
<td>Wab1-R</td>
<td>33</td>
<td>0.0</td>
<td>0.690</td>
</tr>
<tr>
<td></td>
<td><em>lg1-R</em></td>
<td>48</td>
<td>0.0</td>
<td>0.324</td>
</tr>
<tr>
<td></td>
<td><em>lg1-R; Wab1-R</em></td>
<td>67</td>
<td>0.0</td>
<td>0.672</td>
</tr>
</tbody>
</table>

Analysis of the data suggested a relationship between sector position and effect on leaf width. In both Wab1-R and *lg1-R; Wab1-R* plants, sectors near the midrib were not associated with a significant difference in leaf-half width, whereas leaf-halves with sectors in lateral and marginal positions were significantly wider than non-sectored leaf halves (Table 3.5 A).

Many of the widest sectors in *lg1-R; Wab1-R* plants were yellow, *v4-R* sectors. To test if there is a difference in behaviour between *v4-R* and *w3-R* sectors, the median difference in leaf-half widths was evaluated separately for yellow and white sectors in *lg1-R; Wab1-R* and *lg1-R* plants. Sectors near the midrib were not included in this analysis as we had previously determined that they have no significant effect on leaf width. Surprisingly, yellow sectors had a significantly greater effect on leaf width than the white sectors (Table 3.5 B). No difference was detected between yellow and white sectors in *lg1-R* controls, indicating that
this effect is not inherent to v4-R sectors, but only occurs in a Wab1-R background.

Table 3.5. Median differences in wab1/- sectored and non-sector ed leaf-half widths, and effect of sector position. The sectored and non-sector ed sides of each leaf were measured from midrib to margin at the blade midpoint. The width of the non-sector ed leaf-half was subtracted from that of the sectored half to give an absolute difference in leaf-half width. Differences in leaf-half widths were analysed using a non-parametric 1-sign test to determine if the median values were significantly different than 0 at the 0.05 confidence level. (A) The relative lateral position of each sector is represented as the distance from the midrib to the closest sector border, divided by the width of the entire leaf-half. Values range from 0 for sectors at the midrib to near 1.0 for sectors at the margin. (B) Median differences were calculated separately for white (w3-R) and yellow (v4-R) sectors in lg1-R and lg1-R; Wab1-R leaves. * indicates values that are significantly different from 0 at the 0.05 confidence level.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lateral position of sector</th>
<th>Median difference (mm)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Wild-type</td>
<td>0-1.0</td>
<td>50</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0-1.0</td>
<td>83</td>
<td>1.0</td>
</tr>
<tr>
<td>Wab1-R</td>
<td>0-0.29</td>
<td>19</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.3-1.0</td>
<td>64</td>
<td>1.0</td>
</tr>
<tr>
<td>lg1-R</td>
<td>0-1.0</td>
<td>140</td>
<td>0.0</td>
</tr>
<tr>
<td>lg1-R; Wab1-R</td>
<td>0-1.0</td>
<td>180</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0-0.29</td>
<td>64</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.3-1.0</td>
<td>116</td>
<td>2.0</td>
</tr>
<tr>
<td>B.</td>
<td>0.3-1.0</td>
<td>44</td>
<td>0.0</td>
</tr>
<tr>
<td>lg1-R; w3</td>
<td>0.3-1.0</td>
<td>18</td>
<td>-0.5</td>
</tr>
<tr>
<td>lg1-R; v4</td>
<td>0.3-1.0</td>
<td>77</td>
<td>1.5</td>
</tr>
<tr>
<td>lg1-R; Wab1-R v3</td>
<td>0.3-1.0</td>
<td>39</td>
<td>3.5</td>
</tr>
<tr>
<td>lg1-R; Wab1-R v4</td>
<td>0.3-1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.4 Mosaic analysis of lg1-R

Previous mosaic analyses indicate that Lg1 acts cell-autonomously to specify ligule and auricle tissues (Becraft et al., 1990). My results indicate that Lg1 has a role in positioning of the blade-sheath boundary and promotes lateral growth at the base of the blade. To determine if these effects are cell-autonomous or non-autonomous and to test the hypothesis that Lg1 conditions ectopic auricle tissue in Wab1-R leaves, sectors of albino lg1-R/- tissue were induced in Lg1; wab1 and Lg1; Wab1-R plants (Figure 3.2). From this mosaic analysis, a
total of 66 *lg1-RI*-sectored leaves were identified in 31 *Lg1;wab1* plants and 98 sectored leaves were identified in 44 *Lg1;Wab1-R* plants.

**Effects of *lg1-RI*-sectors on normal and *Wab1-R* ectopic auricle tissue**

Sectors of white, *lg1-RI*-tissue in *Lg1;wab1* and *Lg1;Wab1-R* plants were scored for their effects on normal ligule and auricle tissues (Table 3.6). Sectors in *Lg1;Wab1-R* plants were also scored for their effects on ectopic auricle and sheath tissue. Sectors through the auricle had either auricle or blade characteristics. Sectors through the ligule were scored as normal, missing or reduced. Missing ligules were completely deleted within sectors. Reduced ligules were present, but the ligule was shorter within the sector than in surrounding tissue.

**Table 3.6. Summary of *lg1-RI*-sector phenotypes.** *lg1-RI*-sectors in *wab1* (A) and *Wab1-R* (B) leaves were scored for layer composition and tissue identity. Sectors were scored as “Bl” if they displayed characteristics of blade tissue, “Aur” if they exhibited auricle characteristics and “Sh” if they exhibited sheath characteristics. Sectors through the ligule were scored as “N” if the ligule appeared normal, “R” if the ligule was reduced in height within the sector and “M” if the ligule was completely missing within the sector.

<table>
<thead>
<tr>
<th>Number of scorable sectors</th>
<th>Sector phenotype</th>
<th>All sector types</th>
<th>white L1/white L2</th>
<th>green L1/white L2</th>
<th>white L1/mixed L2</th>
<th>green L1/mixed L2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. <em>wab1</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auricle</td>
<td>31</td>
<td>Aur</td>
<td>22 (71%)</td>
<td>1</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bl</td>
<td>9 (29%)</td>
<td>1</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Ligule</td>
<td>33</td>
<td>N</td>
<td>15 (46%)</td>
<td>1</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>6 (18%)</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>12 (36%)</td>
<td>2</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>B. <em>Wab1-R</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auricle</td>
<td>67</td>
<td>Aur</td>
<td>21 (31%)</td>
<td>3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bl</td>
<td>46 (69%)</td>
<td>14</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>Ligule</td>
<td>74</td>
<td>N</td>
<td>24 (32%)</td>
<td>5</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>13 (18%)</td>
<td>3</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>37 (50%)</td>
<td>9</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Ect auricle</td>
<td>35</td>
<td>Aur</td>
<td>7 (20%)</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bl</td>
<td>28 (80%)</td>
<td>10</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Ect sheath</td>
<td>9</td>
<td>Sh</td>
<td>5 (56%)</td>
<td>1</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bl</td>
<td>4 (44%)</td>
<td>1</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>
In *wab1* plants, 71% (22/31) of *lg1-R/-* sectors that passed through the auricle had normal auricle characteristics (Table 3.6 A). Fourteen of these sectors had all white, *lg1-R/-* mesophyll layers. This result differs from the findings of Becraft *et al.* (1990), who found that sectors with all white *lg1-R/-* mesophyll layers developed blade characteristics in all (251/251) cases. Four sectors that had white epidermis were scorable for ligule characteristics. In three cases the ligule was missing within the sector, while in one case the ligule appeared normal. Surprisingly, the about half (14/29) of the sectors with green epidermis had ligules that were reduced or missing. This differs from the results of Becraft *et al.* (1990), who found that green, *Lg1* epidermal tissue was strictly correlated with the development of a normal-looking ligule.

In *Wab1-R* plants, 67 *lg1-R/-* sectors were scorable for their effect on normal auricle tissue (Table 3.6 B). Of these, 31% (21/67) had auricle characteristics and 69% (46/67) had blade characteristics. The majority (41/50) of sectors with white mesophyll had blade characteristics, whereas, the majority (12/17) of sectors with some green mesophyll had auricle characteristics. In *Wab1-R* plants, 74 sectors were scorable for ligule characteristics. The majority of these (50/74) had ligules that were reduced or missing.

Only *lg1-R/-* sectors that traversed ectopic auricle tissue in *Wab1-R* leaves could be scored for their effect on this mutant phenotype. Thus, only 35 sectors were scorable for ectopic auricle identity. Sectors adjacent to ectopic auricle tissue were analysed for phenotypic expression (blade or auricle identity) and cell layer composition (green *Lg1/Lg1-R* or albino *lg1-R/-*) (Table 3.6 B). Of the sectors that were scorable for ectopic auricle, most (80%) showed normal blade characteristics within the sector. An example of a *lg1-R/-* sector with blade characteristics is shown in Figure 3.7. The sectored tissue is thinner than the surrounding ectopic auricle tissue, causing buckling of the leaf (Figure 3.7 A). Sections through the sectored region show that the surrounding green *Lg1/Lg1-R* tissue has large cells and long hairs without multicellular bases characteristic of auricle tissue. White *lg1-R/-* tissue is thinner and blade-like (Figure 3.7 B, C).

All (10/10) of the sectors that had all white cell layers, and most (18/21) of the sectors with white mesophyll and green epidermis, had normal blade identity.
(Figure 3.7 B). In contrast, all (4/4) of the sectors with green mesophyll layers had auricle characteristics. No examples of conversion of ectopic auricle to sheath tissue were observed.

Figure 3.7. Phenotype of Ig1-RI- sector in Lg1;Wab1-R leaf. (A) Wab1-R leaf with white, Ig1-RI- sector that traverses ectopic auricle tissue. (B) Hand-section through sector boundary at position indicated by upper arrowhead. Green Lg1 tissue has long hairs without multicellular bases characteristic of auricle tissue. White Ig1-RI- tissue has bulliform cells and prickle hairs characteristic of blade tissue. (C) Hand-section through sectord region indicated by lower arrowhead. Green tissue is thick and auricle-like, with large cells and long hairs. White Ig1-RI- tissue is thinner, with smaller cells. Scale bars = 200μm.

Effects of Ig1-RI- sectors on Wab1-R ectopic sheath tissue

In Wab1-R plants, only Ig1-RI- sectors that traversed ectopic sheath tissue were scorable for this characteristic. Sectors were analysed for phenotypic expression and cell layer composition. Of the nine scorable sectors, 5 (56%) sectors had sheath identity and 4 had blade identity (Table 3.6B). Only two completely white (Ig1-RI-) sectors were obtained. One of these had sheath-like characteristics, while the other had normal blade characteristics. Of the seven sectors with green epidermis and white internal layers, 4 had sheath-like characteristics, while 3 had the characteristics of blade tissue. No sectors were obtained that were scorable for ectopic sheath and had green internal cell layers.
Effects of $lg1$-RI- sectors on leaf width

We hypothesised that if $Lg1$ promotes lateral growth in a cell-autonomous manner, then $lg1$-RI- sectors should undergo less lateral growth than surrounding green tissue. Therefore, leaf halves with sectors should be narrower than non-sectored leaf-halves. The sectored and non-sectored halves of each leaf were measured from midrib to margin at the blade-sheath boundary and at the blade mid-point. Measurements were made of 66 $wab1$ leaves with $lg1$-RI- sectors and 96 $Wab1-R$ leaves with $lg1$-RI- sectors. Fewer leaves were measured at the blade midpoint than at the base of the blade, as some sectors intersect the margin of the leaf before they reach the blade midpoint. No significant difference was found in the width of sectored and non-sectored leaf-halves in either the $wab1$ or $Wab1-R$ backgrounds (Table 3.7).

Table 3.7. Median differences in the width of $lg1$-RI- sectored and non-sectored leaf-halves. The sectored and non-sectored sides of each leaf were measured from midrib to margin at the blade midpoint and the base of the blade. The width of the non-sectored leaf-half was subtracted from that of the sectored half to give an absolute difference in leaf-half widths. A value greater than 0 indicates that the sectored half of the leaf is wider than the non-sectored half. The data were analysed using a non-parametric 1-sign test to determine if the median values were significantly different than 0 at the 0.05 confidence level.

<table>
<thead>
<tr>
<th>Position of measurement</th>
<th>Genotype</th>
<th>N</th>
<th>Median difference (mm)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid-blade</td>
<td>$wab1$</td>
<td>63</td>
<td>0.0</td>
<td>0.775</td>
</tr>
<tr>
<td></td>
<td>$Wab1-R$</td>
<td>91</td>
<td>0.0</td>
<td>0.289</td>
</tr>
<tr>
<td>Base blade</td>
<td>$wab1$</td>
<td>66</td>
<td>0.0</td>
<td>0.890</td>
</tr>
<tr>
<td></td>
<td>$Wab1-R$</td>
<td>96</td>
<td>0.0</td>
<td>0.320</td>
</tr>
</tbody>
</table>

3.3.5 Clonal analysis of $Wab1-R$ leaves

$Wab1-R$ leaves are narrower than wild-type leaves (Hay and Hake, 2004). To investigate the nature of the narrow leaf phenotype, a cell lineage analysis was undertaken. Clonal sectors of white ($lw$-RI-) cells were induced in $Wab1-R$ plants and wild-type ($wab1$) controls. 37 sectored leaves were obtained in 23 wild-type plants and 81 sectored leaves were obtained in 32 $Wab1-R$ plants. Eleven sectors were recorded in the culm of wild-type plants, and 28 sectors in $Wab1-R$ plants.
Leaves were measured from midrib to margin at the blade midpoint and at the base of the blade. *Wab1-R* leaves were significantly narrower than wild-type leaves (Table 3.8). The difference in width was more pronounced at the blade midpoint than at the base of the blade.

Sectors were assigned to one of three lateral positions within the leaf blade; midrib (0-0.29), lateral (0.3-0.59) or marginal (0.6-1.0). Mean sector widths in *Wab1-R* and wild-type leaves were compared separately for sectors in each lateral position (Table 3.8). At the blade midpoint, mean sector widths in midrib and lateral domains were not significantly different for *Wab1-R* and wild-type leaves. However, only three lateral position sectors were obtained in wild-type leaves and these were notably wider than sectors in *Wab1-R* leaves. Sectors in the marginal part of the leaf were significantly wider in wild-type leaves than in *Wab1-R* leaves. At the base of the blade, midrib domain sectors were significantly wider in *Wab1-R* leaves than in wild-type leaves. There was no significant difference in the width of *Wab1-R* and wild-type lateral or marginal domain sectors measured at the base of the blade.

Table 3.8. Mean widths of clonal sectors in *Wab1-R* and wild-type leaves. Sector width and leaf-half width were measured at the blade midpoint and the base of the blade. Mean sector widths were calculated separately for sectors near the midrib (sector position 0-0.29), in the lateral domain (0.3-0.59), and near the margin (0.6-1.0). Wild-type and *Wab1-R* means were compared by Student’s T-test to determine if they are significantly different at the 0.05 confidence level. * indicates values that are significantly different. Sample sizes for each category are indicated in brackets. SE = standard error.
Hay and Hake (2004) hypothesised that the narrowness of Wab1-R leaves is due to the deletion of a lateral domain. If this hypothesis is correct, then we would predict that sectors that originate in this particular region of the SAM would not extend into the leaf blade. The culm is not affected by Wab1-R and, since it is a radial structure, the position of clonal sectors in the culm approximates the position of sectors in the SAM from which it is derived (Scanlon, 2000). Therefore, to determine if a lateral domain is deleted in Wab1-R leaves, the position of each clonal sector in the culm was recorded and its position calculated in degrees from the midrib (Figure 3.8). It was then noted whether the sector continued into the leaf above.

From the Wab1-R clonal analysis, sectors were obtained that covered all radial positions of the culm except the region between 0° and 31°. All sectors that were observed in the culm continued into the blade of the leaf above. These results do not support the hypothesis that the Wab1-R narrow leaf phenotype is caused by the deletion of a lateral domain.

![Figure 3.8. Calculation of radial position of clonal sectors in the culm. The position in the culm corresponding to the midrib of the leaf above was designated 0°. Measurements were made from 0° to the inner boundary of the sector (dashed line), from 0° to the outer sector boundary, and of the entire culm circumference. The radial position of the inner and outer sector boundaries were then calculated in degrees from the midrib (see text).](image)

### 3.3.6 **Wab1-R leaves have fewer lateral veins by plastochron 4**

Hay and Hake (2004) found that Wab1-R leaves are narrower and have fewer lateral veins than wild-type leaves. To determine when the reduction in lateral veins is first apparent, lateral veins were counted in transverse sections of
Wab1-R and wild-type (B73) leaf primordia. My data show no significant difference in the number of lateral veins in Wab1-R and wild-type leaf primordia at stages P1, P2 or P3 (Figure 3.9). At P4, there was a significant difference in the number of lateral veins in Wab1-R and wild-type leaf primordia. Wild-type leaf primordia at P4 had an average of 15.3 lateral veins, whereas Wab1-R leaf primordia at the same stage had an average of 13.4 lateral veins. At P5 the difference was even more pronounced – wild-type leaf primordia had an average of 21.4 lateral veins, whereas Wab1-R leaf primordia had an average of 16.6 lateral veins.
A. Lateral vein number in wild-type and Wab1-R leaf primordia

![Graph showing lateral vein number in wild-type and Wab1-R leaf primordia]

B. Table showing plastochron, wild-type (B73), Wab1-R, and P-value.

<table>
<thead>
<tr>
<th>Plastochron</th>
<th>Wild-type (B73)</th>
<th>Wab1-R</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Mean SE 0.4</td>
<td>0.1</td>
<td>0.225</td>
</tr>
<tr>
<td></td>
<td>0.2 (N=8)</td>
<td>0.1 (N=9)</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>Mean SE 3.2</td>
<td>3.3</td>
<td>0.878</td>
</tr>
<tr>
<td></td>
<td>0.6 (N=12)</td>
<td>0.6 (N=11)</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>Mean SE 8.8</td>
<td>9.1</td>
<td>0.752</td>
</tr>
<tr>
<td></td>
<td>0.7 (N=12)</td>
<td>0.4 (N=16)</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>Mean SE 15.3</td>
<td>13.4</td>
<td>0.031*</td>
</tr>
<tr>
<td></td>
<td>0.6 (N=8)</td>
<td>0.5 (N=14)</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>Mean SE 21.4</td>
<td>16.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>0.4 (N=5)</td>
<td>0.8 (N=8)</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>Mean SE 26</td>
<td>18</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>0.8 (N=4)</td>
<td>0.6 (N=3)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.9. Lateral vein number in wild-type and Wab1-R leaf primordia.

(A) Lateral veins were counted in wild-type (B73) and Wab1-R leaf primordia at stages P1-P6. The mean number of lateral veins was calculated separately for wild-type and Wab1-R leaf primordia at each plastochron. Mean number of lateral veins in wild-type and Wab1-R leaf primordia is plotted against plastochron number. Bars represent standard error.

(B) The wild-type and Wab1-R means for each plastochron were compared by Student's t-test to determine if they are significantly different at the 0.05 confidence level. * indicates values that are significantly different. SE = standard error.
3.4 Discussion

In the maize leaf, ligule and auricle form at the boundary between the blade and sheath. *Lg1* has been implicated in the propagation of a signal that correctly positions this boundary, and is necessary for the development of ligule and auricle tissues. The dominant *Wab1-R* mutation affects medial-lateral and proximal-distal patterning, resulting in narrow leaves and inappropriate cell differentiation. The recessive *lg1-R* mutation exacerbates the *Wab1-R* phenotype. *Lg1* expression is activated precociously in *Wab1-R* leaves and may counteract some effects of the *Wab1-R* mutation (Foster et al., 2004). A mosaic analysis of *Wab1-R* was conducted in *Lg1* and *lg1-R* backgrounds to determine if *Wab1-R* affects leaf development in a cell-autonomous manner. This analysis showed that *Wab1-R* generally acts cell-autonomously to disrupt proximal-distal patterning. Examples of *Wab1-R* non-autonomy were only observed in a *Lg1* background, supporting a role for *Lg1* in signal propagation. A mosaic analysis of *lg1-R* in a *Wab1-R* background indicated that *Wab1-R* ectopic auricle tissue is conditioned by *Lg1*. The mosaic analyses and comparison of mutant leaf shapes revealed previously unreported functions of *Lg1* in both normal leaf development and in the dominant *Wab1-R* mutant. A detailed discussion of these results is presented below.

3.4.1 *Lg1* influences cell-autonomy of the *Wab1-R* phenotype

In the majority of *Wab1-R* leaves and in all *lg1-R; Wab1-R* leaves, scorable *wab1/-* sectors exhibited characteristics of normal blade tissue, whereas adjacent tissue differentiated inappropriately as sheath or auricle. The sharp boundaries between tissue types were coincident with sector boundaries, indicating that *Wab1-R* generally acts cell autonomously in the lateral dimension to disrupt normal proximal-distal patterning.

Twelve of the 51 scorable sectors showed some aspect of the *Wab1-R* mutant phenotype and thus are exceptions to the general rule of cell-autonomy. Of these 12, two were completely albino and therefore had no layer with *Wab1-R*. Of the 10 remaining sectors, half carried *Wab1-R* only in the epidermis, and half carried *Wab1-R* in the epidermis and/or one mesophyll layer. Thus, *Wab1-R*
may act non-autonomously in the lateral dimension and/or between cell layers to influence cell identity in \textit{wab1/-} tissue.

In contrast, all \textit{wab1/-} sectors in \textit{lg1-R;Wab1-R} plants had normal cell types. \textit{Wab1-R} in either the epidermis or a single mesophyll layer did not condition the mutant phenotype (12 of 26 sectors). These results suggest that normal \textit{Lg1} function is required for \textit{Wab1-R} to act non-autonomously.

A few cases of non-autonomy were observed in which the normal blade phenotype (\textit{wab1}) was seen on the margin side of the sector as well as within the sector (Figure 3.5 A, B). The extension of the normal phenotype from the sector into genetically \textit{Wab1-R} cells was only seen in mildly affected plants. This pattern could reflect the fact that the \textit{Wab1-R} phenotype is most severe in the lateral domain. Alternatively, it may be that once correct proximal-distal patterning is established within \textit{wab1/-} tissue, this information can be propagated towards the margins into \textit{Wab1-R/wab1} tissue, but only if \textit{Wab1-R} activity is low. Interestingly, non-autonomy was never documented in \textit{lg1-R;Wab1-R} plants. Cells in \textit{lg1-R;wabl/-} sectors always had blade identity and cells outside of these sectors always had sheath identity. Thus, \textit{Lg1} may affect the autonomy of \textit{Wab1-R} in both lateral and transverse dimensions.

Previous mosaic analyses of \textit{lg1-R} have indicated that \textit{Lg1} is involved in signal propagation, while also acting cell-autonomously to induce ligule and auricle (Becraft \textit{et al.}, 1990; Becraft and Freeling, 1991). One of the key findings of this work was the observation that ligule and auricle reinitiated immediately within \textit{Lg1/lg1-R} tissue on the margin side of \textit{lg1-R/-} sectors, but was displaced proximally. The authors interpreted this as evidence that \textit{Lg1} is involved in the propagation of a “make ligule and auricle” signal, and that this signal moves from the midrib towards the margins. Observations from the current analysis support this hypothesis.

These data suggest that \textit{Wab1-R} alters positional information in a cell-autonomous manner, resulting in inappropriate cell differentiation. We speculate that \textit{Lg1} is responsible for the non-autonomous effects of \textit{Wab1-R} that were observed in our mosaic analysis. According to this model, \textit{Lg1} may occasionally
transmit the signal to initiate ectopic auricle from Wab1-R tissue into wab1/-sectors (Figure 3.10 A). Similarly, Lg1 may relay correct positioning of the auricle and ligule from wab1/-sectors into adjacent Wab1-R tissue. In the absence of Lg1, there is no lateral signalling from Wab1-R tissue into wab1/-sectors, or from sectors into Wab1-R tissue (Figure 3.10 B).

![Diagram](A. Wab1-R B. Ig1-R;Wab1-R)

**Figure 3.10. Lg1 affects cell-autonomy of the Wab1-R phenotype.** (A) Cartoon illustrating spread of the Wab1-R phenotype (wavy lines) into a wab1/-sector and spread of normal phenotype (no wavy lines) from a wab1/-sector into Wab1-R/wab1 tissue. We propose that Lg1 can transmit both correct and incorrect positional signals towards the leaf margins (red arrows). (B) In the absence of Lg1, the Wab1-R phenotype is strictly cell-autonomous.

### 3.4.2 Loss of Wab1-R is associated with an increase in leaf width

A significant difference was found between the widths of sectored (i.e. cells that have lost Wab1-R) and non-sectored leaf-halves in Wab1-R and Ig1-R;Wab1-R plants, but not in wild-type or Ig1-R control plants. No obvious differences in cell width were seen between similar cell types in sectored and non-sectored tissue (data not shown). Therefore, it can be inferred that the increase in lateral growth associated with wab1/-sectors was the result of increased longitudinal cell divisions. It is difficult to determine if increased cell division was confined to the sectored tissue or occurred throughout the sectored leaf-half. The fact that a significant difference was recorded between sectored and non-sectored leaf-half widths suggests that Wab1-R restricts lateral growth with at least some degree of autonomy. If Wab1-R were entirely non-autonomous, then the presence of a wab1/-sector would have no effect on leaf-half width. A mosaic analysis of the tangled mutation in maize found that the cell division defect is autonomous in both the lateral and transverse dimensions (Walker and Smith,
Thus, there is precedence for strict autonomy of cell division patterns in chimeric tissues.

In both *lg1-R; Wab1-R* and *Wab1-R* leaves, sectors positioned near the midrib had no significant effect on leaf-half width, whereas sectors in the outer two thirds of the leaf-half were associated with significant differences between sectored and non-sectored leaf-half widths. This result could reflect the fact that sectors near the midrib tend to be very narrow, and hence have a minimal effect on lateral growth. Alternatively, it may reflect the fact that the *Wab1-R* phenotype primarily affects regions outside the midrib domain (Hay and Hake, 2004).

### 3.4.3 The role of *Lg1* in leaf morphogenesis

This study provides evidence of previously unreported functions of *Lg1* in leaf morphogenesis. *lg1-R* leaves were found to have longer sheaths and shorter blades than wild-type (*Lg1/lg1-R*) siblings, although overall leaf length was not significantly different. Thus, the blade-sheath boundary was shifted distally in *lg1-R* leaves (Figure 3.11 B). These results suggest that *Lg1* is required for correct positioning of the blade-sheath boundary.

It was also determined that *lg1-R* leaves were narrower at the base of the blade than wild-type siblings (Figure 3.11 B). A comparison of clonal sectors in *lg1-R* and wild-type control plants indicated that margin and lateral sectors were significantly narrower in *lg1-R* mutants than in wild-type leaves. These results imply that *Lg1* promotes lateral growth at the base of the blade. Sylvester and co-workers (1990) showed that a localised increase in both longitudinal and transverse anticlinal divisions generates a band of small cells across the base of the leaf primordium. This preligule band is a necessary prerequisite for the formation of ligule and auricle. *lg1-R* mutants are specifically defective in longitudinal divisions in the preligule region and at the base of the blade. *Lg1* may promote lateral growth via a direct effect on the rate and orientation of cell divisions. Alternatively, the development of the auricle itself may drive lateral growth of the lower leaf blade, ensuring the coordinated expansion of the leaf.
We suggest that misexpression of Lg1 is responsible for the ectopic auricle and much of the lateral growth at the base of Wab1-R leaf blades. This is reflected in the shape of Wab1-R leaves, which are relatively wide at the base of the blade, but narrow in more distal areas (Figure 3.11 C). We speculate that the lateral signalling function of Lg1 permits some recovery of proximal-distal patterning at the margins of Wab1-R leaves (Figure 3.11 C). In the absence of Lg1, Wab1-R leaves never establish blade in this region, and are extremely narrow (Figure 3.11 D).

![Figure 3.11. Model for Lg1 function in leaf morphogenesis.](image)

(A) Cartoon of wild-type leaf illustrating distal blade (green) and proximal sheath (blue) separated by ligule and auricle (red). (B) In the absence of Lg1, ligule and auricle are deleted, the blade-sheath boundary is shifted distally, and the base of the blade is narrower than in wild-type leaves. (C) Wab1-R disrupts proximal-distal patterning and restricts lateral growth of the blade. Misexpression of Lg1 in Wab1-R leaves results in ectopic ligule and auricle, and partially compensates for the narrow leaf phenotype at the base of the blade. The lateral signalling function of Lg1 permits some recovery of proximal-distal patterning at the margins of Wab1-R leaves. (D) In the absence of Lg1, Wab1-R leaves never establish blade at the margins and are extremely narrow.

### 3.4.4 Ectopic auricle tissue in Wab1-R leaves is conditioned by Lg1

To further investigate interactions between Lg1 and Wab1-R, a mosaic analysis of lg1-R was conducted in a Wab1-R background. Ig1-RI- sectors were analysed for their effects on ectopic auricle and sheath tissue. The majority
(28/35) of Ig1-R/ sectors in Wab1-R leaves that were scorable for ectopic auricle tissue exhibited normal blade characteristics. In these cases, ectopic auricle tissue ended abruptly at sector boundaries (Figure 3.7). The results of this analysis suggest that Lg1 acts cell-autonomously in the lateral dimension to specify ectopic auricle tissue in Wab1-R mutants and supports the hypothesis that misexpression of Lg1 is responsible for the ectopic auricle tissue in Wab1-R leaf blades.

All (10/10) of the sectors that were completely white and traversed ectopic auricle tissue had blade identity (Table 3.6 B). This result suggests that Lg1 acts strictly cell-autonomously in the lateral dimension to condition ectopic auricle in Wab1-R leaves. Where Lg1 was present only in the epidermis, the majority (18/21) of sectors that traversed ectopic auricle tissue had blade identity. The observation that a small number (3/21) of such sectors exhibited auricle characteristics may indicate that there is some Lg1-mediated signalling between the epidermis and internal cell layers. However, there are alternative explanations. The Ig1-R phenotype varies in expressivity, thus these sectors may reflect a lack of expressivity rather than non-autonomy by Lg1 (Becraft et al., 1990). Alternatively, they could be a result of recombination events between Lg1 and al-R. Becraft et al. (1990) estimated that there may be 7% recombination between Ig1-R and al-R. The current data do not allow these possibilities to be distinguished.

Only four sectors with internal layers of green, Lg1 cells were obtained that were scorable for the ectopic auricle phenotype (Table 3.6 B). All of these sectors had auricle identity. This result suggests that Lg1 in internal cell layers may act non-autonomously to condition the Wab1-R ectopic auricle phenotype in overlying cell layers. However, more sectors would need to be analysed to confirm this. The result is consistent with the work of Becraft et al. (1990), who found that Lg1 in any cell layer is sufficient to induce the development of normal auricle in all layers.

The Wab1-R mutation causes distal tissues to differentiate as more proximal tissue types (Hay and Hake, 2004). In a Lg1 background, ectopic auricle and sheath-like tissues develop in the leaf blade. In the absence of Lg1 most of the
blade is converted to sheath-like tissue. \( Lg1 \) has previously been shown to specify auricle tissue (Becraft et al., 1990). We hypothesised that the primary \( Wab1-R \) defect is the "proximalisation" of distal tissues, and that ectopic auricle tissue in \( Wab1-R \) mutants is conditioned through ectopic expression of \( Lg1 \). If this hypothesis is correct, then, in the absence of \( Lg1 \), sheath would be the "default" tissue type. This hypothesis predicts that \( lg1-R/l- \) sectors that traverse regions of ectopic auricle will have sheath identity.

In the mosaic analysis of \( lg1-R \) in \( Wab1-R \) plants, the majority (28/35) of sectors that passed through ectopic auricle tissue had blade identity, the remainder were auricle-like (Table 3.6 B). No examples of conversion of ectopic auricle to sheath tissue were observed. These data are consistent with the hypothesis that ectopic auricle in \( Wab1-R \) mutants is conditioned through \( Lg1 \). However, they do not support the hypothesis that sheath tissue is the default tissue type in the absence of \( Lg1 \) function.

The results of this mosaic analysis of \( lg1-R \) in \( wab1 \) leaves differed from those of previous analyses (Becraft et al., 1990). Becraft et al. (1990) found that sectors with all white (\( lg1-R/l- \)) mesophyll layers had blade characteristics in all (251/251) cases. In our experiment, 14/22 sectors with all white mesophyll layers had auricle characteristics (Table 3.6 A). There are a number of possible explanations for this result. Firstly, there may have been a recombination event between \( al-R \) and \( Lg1 \), such that white sectors in these leaves were \( Lg1 al-R/a-R\) rather than \( lg1-R al-R/l- \). Secondly, the genetic stock may have been heterozygous for a second, unlinked albino marker gene. This second marker may have been uncovered in some plants, thus creating albino sectors that were \( Lg1/lg1-R \). Finally, white sectors with auricle characteristics may reflect the variable expressivity of the \( lg1-R \) phenotype. Vestiges of ligule and auricle often occur on upper leaves of \( lg1-R \) plants (Becraft et al., 1990; Sylvester et al., 1990). Five of the sectors we observed with auricle characteristics occurred on leaf ten or lower, suggesting that these were not the result of incomplete expressivity of the \( lg1-R \) phenotype. The results of this analysis also differed from those of Becraft et al. in that about half (14/29) of the sectors with green (\( Lg1/lg1-R \)) epidermis had ligules that were reduced or missing. Becraft et al. (1990) found that green, \( Lg1 \) epidermal tissue was strictly correlated with the
development of a normal-looking ligule. It is not clear why our result differed from this analysis.

3.4.5 Effects of *lg1-RI*- sectors on *Wab1-R* ectopic sheath tissue

Nine sectors were obtained that were scorable for ectopic sheath-like tissue (Table 3.6 B). Seven had white (*lg1-RI-*) mesophyll with green (*Lg1/lg1-R*) epidermis and two were entirely white. Of these, four had blade identity and five had sheath-like characteristics. *lg1-R* leaves have a less defined blade-sheath boundary than wild-type leaves, and *lg1-R;Wab1-R* double mutants have more extensive regions of sheath tissue extending into the leaf blade than *Wab1-R* single mutants (Becraft *et al.*, 1990). Therefore, it was not predicted that *lg1-RI*-sectors would restore blade-like characteristics. The fact that half of the observed sectors had sheath-like identity indicates that ectopic sheath tissue is not dependent on *Lg1* function. Some areas of ectopic sheath tissue did end at *lg1-RI-* sector boundaries. This may reflect the fact that clonal sectors in maize leaves and regions of cleared tissue in *Wab1-R* leaves both tend to be bordered by lateral veins (Cerioli *et al.*, 1994). Thus, it is likely that clonal sectors were simply coincident with boundaries of ectopic sheath tissue.

3.4.6 *Lg1* promotes lateral growth in a non-cell autonomous manner

The analysis of *lg1-R* leaf shape indicates that *Lg1* promotes lateral growth of leaves in both *wab1* and *Wab1-R* backgrounds. To determine if *Lg1* promotes lateral growth in a cell-autonomous manner, *Lg1;Wab1-R* and *Lg1;wab1* leaves with *lg1-RI-* sectors were measured at the blade midpoint and the base of the blade.

It was predicted that if *Lg1* promotes lateral growth cell-autonomously, then leaf-halves with *lg1-RI-* sectors should be narrower than non-sectored halves of the same leaves. Analysis of measurement data showed no significant difference in the width of sectored and non-sectored leaf-halves in either the *wab1* or *Wab1-R* backgrounds (Table 3.7). Thus, the data do not support the hypothesis that *Lg1* promotes lateral growth in a cell-autonomous manner, suggesting that *Lg1* acts non-autonomously to promote lateral growth.
3.4.7 *Lg1* has both cell-autonomous and non-autonomous functions

Previous mosaic analyses have shown that *Lg1* acts cell-autonomously to specify auricle and ligule (Becraft *et al.*, 1990; Becraft and Freeling, 1991). The current study indicates that *Lg1* also conditions *Wab1-R* ectopic auricle tissue in a cell-autonomous manner. However, our data suggest that *Lg1* promotes lateral growth in a non-autonomous manner. Thus, *Lg1* has both cell-autonomous and non-autonomous functions. In addition to specifying ligule and auricle tissue, there is evidence that *Lg1* is required for correct positioning of the blade-sheath boundary. The blade-sheath boundary of *Lg1* leaves is less defined and is shifted distally (Becraft *et al.*, 1990). These results point to multiple roles for *Lg1* in leaf morphogenesis and tissue specification, which may be mediated by multiple downstream pathways.

3.4.8 *Wab1-R* leaf primordia are narrower and initiate fewer lateral veins than wild-type leaf primordia

Hay and Hake (2004) found that *Wab1-R* leaf blades are much narrower than wild-type and have fewer lateral veins. Sheath width is not affected by *Wab1-R*. The lateral growth defect was seen in leaf primordia as young as P3. Wild-type P3 leaf primordia completely enclosed the SAM and younger leaf primordia, whereas, *Wab1-R* leaf primordia at the same stage did not fully enclose the apex (Hay and Hake, 2004). To determine when the reduction in lateral vein number is first apparent, lateral veins were counted in early leaf primordia of *Wab1-R* and wild-type plants. My results show no significant difference in *Wab1-R* and wild-type lateral vein number in P3 stage primordia (Figure 3.9). At P4, *Wab1-R* leaf primordia have fewer lateral veins than wild-type. Thus, the difference in lateral vein number is apparent one plastochron later than the visible reduction in width. This finding is consistent with a model in which primordium size governs the number of veins that are initiated. It has been proposed that vascular pattern is determined by a self-organising system involving auxin transport (Scarpella *et al.*, 2006).
Hay and Hake (2004) hypothesised that the \textit{Wab1-R} narrow leaf phenotype is due to the deletion of a lateral domain in the leaf blade. To test this hypothesis, a clonal analysis was conducted in \textit{Wab1-R} and wild-type leaves. A similar clonal analysis of \textit{ns} leaves found that sectors derived from a specific region of the \textit{ns} meristem did not extend into the leaf above (Scanlon and Freeling, 1997). This study provided evidence that a lateral domain encompassing the leaf margins is not initialised in \textit{ns} mutants.

It was predicted that if \textit{Wab1-R} causes the deletion of a specific lateral domain in the leaf, then sectors that are present in the corresponding radial position in the SAM would not extend into the leaf blade. The position of sectors within the culm was used to approximate their radial position within the SAM (Figure 3.8). Overlapping sectors that covered almost all positions of the culm were obtained. All of these sectors continued into the leaf blade. Thus, these results do not support the hypothesis that \textit{Wab1-R} causes the deletion of a lateral domain. No culm sectors were obtained in the region nearest the midrib. Therefore, the possibility that this region is affected by \textit{Wab1-R} cannot be ruled out. This seems unlikely, however, as the cells immediately adjacent to the midrib contribute little to the overall width of the leaf (Poethig and Szymkowiak, 1995), and \textit{Wab1-R} has no obvious effect on tissue identity in this region.

We suggest that \textit{Wab1-R} causes a reduction in lateral growth, rather than the deletion of a specific domain. A comparison of clonal sector width in \textit{Wab1-R} and wild-type leaves provides support for this hypothesis, although the experiment did not generate enough sectored leaves to undertake a detailed comparison of cell fate in \textit{Wab1-R} and wild-type leaves. The greatest difference in the width of \textit{Wab1-R} and wild-type leaves was seen at the blade midpoint (Table 3.8). Sectors in the marginal domain of \textit{Wab1-R} leaves were significantly narrower than sectors in the marginal domain of wild-type leaves. There was also a notable difference in the width of lateral domain sectors measured at the blade midpoint in \textit{Wab1-R} and wild-type leaves, although this difference was not found to be statistically significant. This is likely to reflect the small number of sectors obtained in this region.
At the base of the blade, sectors in the midrib domain of Wab1-R leaves were wider than sectors in the equivalent position in wild-type leaves. This result was surprising, as Wab1-R leaves are slightly narrower than wild-type leaves at this point, although the reduction is less severe than at the blade midpoint. No significant difference was found in the width of Wab1-R and wild-type lateral and marginal domain sectors measured at the base of the blade. This may reflect the fact that the narrow leaf phenotype is much less severe at the base of the blade than at the blade midpoint (Hay and Hake, 2004). This finding is consistent with the hypothesis that ectopic Lg1 expression in Wab1-R leaves promotes lateral growth at the base of the blade and partially compensates for the Wab1-R narrow leaf defect.
4. *milkweed pod1-R*

4.1 Introduction

*milkweed pod1-R* (*mwp1-R*) is a recessive mutation that was first identified by ectopic outgrowths on the abaxial surface of the husk leaves. The mutant was discovered by Oliver Nelson and is believed to be a spontaneous mutation (pers. comm., Hector Candela and Sarah Hake). The *Mwp1* gene has recently been cloned by Hector Candela, a postdoctoral fellow in Sarah Hake's lab. *Mwp1* is a member of the KANADI gene family, and *mwp1-R* is the first *kan* loss-of-function mutant to be reported in maize (pers. comm., Hector Candela and Sarah Hake).

The various maize lateral organs exhibit morphological differences which may be viewed as differential elaboration of a common pattern. The vegetative leaf is subdivided into blade and sheath, with the blade being the dominant part, whereas the husk leaves are mainly sheath tissue with a small residual blade at the tip. The prophyll and gynoeicum are each thought to be derived from the fusion of two phytomers. The prophyll is believed to form via the fusion of two primordia along adjacent margins. Each of the midrib regions develops a prominent keel. The *mwp1-R* phenotypes observed in different lateral organs reflect their diverse morphologies.

In order to elucidate the role of *Mwp1* in normal leaf development, a detailed analysis of the *mwp1-R* phenotype and characterisation of the expression patterns of known polarity genes in *mwp1-R* lateral organs were undertaken. In addition, prophyll development was investigated in *mwp1-R* and wild-type backgrounds in order to gain a better understanding of axial patterning in lateral organs with diverse morphologies.
4.2 Specific Materials and Methods

4.2.1 Material for SEM and histology

Detailed protocols for SEM and histology are provided in Sections 2.5 and 2.6.

Tissue from mwp1-R and wild-type husk leaves was fixed for SEM and sectioning. Samples were taken from a point midway between the base and tip and midway between the centre and margin of the leaf.

Samples of mwp1-R and wild-type prophyll tissue were fixed for SEM and sectioning. Tissue was taken from the keel region, midway between the base and tip of the prophyll.

Tissue from unfused mwp1-R prophylls displaying the "tab" phenotype was fixed for SEM and sectioning. Tissue was taken from the "tabs" of mwp1-R prophylls and from the central membrane of wild-type prophylls at a point midway between the base and tip of the prophyll.

Tissue from mwp1-R and wild-type vegetative leaf sheath margins was fixed for SEM and sectioning. Tissue was taken from a point midway between the base of the leaf and the blade-sheath boundary.

Segments of mwp1-R and wild-type silk tissue were fixed for SEM and sectioning. Samples for SEM were taken from the base of the silk. Samples for sectioning were taken at a point midway between the base and tip of the silk.

Tissue from mwp1-R;Wab1-R leaves was fixed for SEM and sectioning. Samples were taken from regions where ectopic sheath-like tissue extended into the leaf blade in mwp1-R;Wab1-R leaves and equivalent positions in wild-type leaves.
4.2.2 Measurements of *mwp1-R* and wild-type lateral organs

Measurements were all made on mature field grown W23 and A188 inbred plants, and on *mwp1-R* introgressed into either W23 or A188 five times. Introgressed material was supplied by Sarah Hake and Hector Candela. Husk leaf, prophyll and vegetative leaf measurements were made using a ruler and a flexible dressmakers' measuring tape. Each data set comprised eight or more individuals (exact sample sizes are given in results tables).

**Prophylls and husk leaves**

The prophyll and husk leaves 1, 4 and 8 (numbered from outer to inner) from the upper ear of each plant were measured. Prophyll and husk leaf length were measured from the base of the leaf to the blade-sheath boundary. Width was measured from margin to margin at a point midway between the base of the leaf and the blade-sheath boundary. For prophylls, the distance between the keels was measured at a point midway between the base and tip of the leaf (see Table 4.1).

**Vegetative leaves**

Vegetative leaves 8, 9 and 10 (counting down from the tassel) were measured. Blade length was measured from the blade-sheath boundary to the tip of the leaf. Sheath length was measured from the base of the leaf to the blade-sheath boundary. Half-leaf width was measured from midrib to margin. Blade width was measured at the widest part at the base of the blade. Sheath width was measured at a point midway between the base of the leaf and the blade-sheath boundary.

**Silks**

Sectioned material was photographed under the stereomicroscope. Measurements were made from calibrated digital photographs. Measurements were made of the distance between the veins and of the distance from one vein to the outer edge of the silk (see Table 4.6).

**Paleae and glumes**

Paleae and glumes were dissected from male florets and flattened onto double sided tape attached to glass microscope slides. They were photographed under
the stereomicroscope. Measurements were made from calibrated digital photographs. Length was measured from base to tip. Width was measured from margin to margin at the widest point. For each palea, the distance between the veins was measured.

Statistical analysis
Mean values for each measurement point were calculated separately for mwp1-R and wild-type. Means were compared by Student's t-test to determine if they were significantly different at the 0.05 confidence level. Data were analysed using Microsoft Office Excel 2003.

4.2.3 SEM of developing prophylls
Replicas of axillary buds were created using the method described in Section 2.5.2. Axillary buds were taken from reproductive nodes. Axillary buds are suppressed in nodes above the ear, so the most distal nodes that contained axillary buds were selected (Poethig, 1988). Subtending leaves were removed to expose axillary buds. Buds at the earliest stages were cast while still attached to the main axis. Later stage buds were detached from the main axis using a razor blade before casting. This allowed the side of the prophyll adjacent to the culm to be viewed.

4.2.4 Measurements of developing prophylls
Developing prophylls were measured from electron micrographs. Overall length was measured from base to tip. When the tips were curled over, a piece of thread was used to trace the line of the prophyll and the length of the straightened thread was measured. The length of the fused region was measured from the base of the prophyll to the cleft where the two prophyll tips join, or to the top of the strip of tissue connecting the two prongs in unfused mwp1-R prophylls (see Figure 4.11).

4.2.5 In situ hybridisation
The in situ hybridisation protocol used is a modified version of the one described by Jackson (1991).
**Probe synthesis**

Clones of the *rld1* and *zyb9* genes in the vector pBluescript (Stratagene) were kindly provided by Marja Timmermans (Cold Spring Harbour Laboratory, New York). The *rld1* clone encompassed nucleotides 619-1674 of the *rld1* coding sequence (Juarez et al., 2004b). The *zyb9* clone comprised the 5' region, including the Zn-finger domain (Juarez et al., 2004a). GenBank accession numbers are: *rld1* AY501430; *zyb9* AY313903.

Clones were transformed into DH5α cells, selected on ampicillin plates and cells were grown in 10 ml overnight cultures. Plasmid DNA was extracted using the QIAprep® Spin Miniprep Kit (QIAGEN) and eluted in 50 μl of water. Plasmid DNA was sequenced by the Alan Wilson Centre Genome Sequencing Service to confirm that the correct sequence had been obtained.

Plasmid was digested with the appropriate restriction enzyme to give a linear template for the transcription reaction. For the *zyb9* antisense probe, plasmid was linearised with BamHI and the T7 RNA polymerase (Roche) was used in the transcription reaction. For the *rld1* antisense probe, plasmid was linearised with Xhol and the T3 RNA polymerase (Roche) was used in the transcription reaction. Protein was removed from the linearised plasmid by phenol chloroform extraction.

DIG-labelled probe was synthesised by *in vitro* transcription using 1 μg of the linearised plasmid as a template, DIG RNA labelling mix (Roche) and the supplied buffer in a final volume of 20 μl. The transcription reaction was carried out at 37°C for 1 h. An aliquot was saved to run on a gel after DNase treatment.

The DNA template was degraded by DNase treatment. Water and 5 μl RNase free DNase (Roche) were added to the transcription reaction to make a final volume of 100 μl and incubated at 37°C for 10 min. An aliquot was run on a gel alongside the reserved aliquot from the transcription reaction to ensure that the template was degraded. Probe was ethanol precipitated, then resuspended in 50 μl of water and 50 μl of formamide to create the stock probe.
RNase treatment of solutions and equipment

Precautions were taken to prevent RNase contamination during the *in situ* hybridisation procedure. All solutions were made using DEPC-treated water (1 ml of DEPC was added to 1 L of milli-Q water, incubated overnight with occasional shaking, and then autoclaved). All glassware, slide racks and forceps were baked at 250°C for 6-8 h. Plastic containers were soaked in H₂O₂ overnight and then rinsed in RNase-free water. Benches were cleaned with RNase away (Molecular BioProducts). Latex gloves were worn for all steps and changed regularly.

Prehybridisation treatments

Slides were placed in metal slide racks and all prehybridisation treatments were carried out at room temperature.

Dewaxing

Slides were dewaxed in two changes of Histoclear for 10 min each.

Hydration

Slides were hydrated through a graded ethanol series (2 x 100% for 2 min, 95%, 85%, 70%, 50%, 30% for 1 min each, 0.85% NaCl for 2 min), then immersed in 0.1 M HCl for 20 min, water for 5 min and 1xPBS for 2 min.

Pronase treatment

Pronase was prepared by predigesting 50mg/ml pronase (Roche) at 37°C for 4 h to remove nucleases. 1 ml aliquots were stored at -20°C.

Pronase solution was prepared immediately before use by adding 1 ml of 50 mg/ml predigested pronase to 400 ml of 37°C pronase buffer (50 mM Tris pH 7.5, 5 mM EDTA). Slides were incubated in pronase solution for 20 min at 37°C. Pronase digestion was stopped by immersing slides in 0.2% (w/v) glycine in 1xPBS for 2 min, then rinsing in 1xPBS for 2 min.

Acetic anhydride

A 400 ml container of 0.1 M triethanolamine was placed on a stir plate and the slide rack was suspended in this solution. 2 ml of acetic anhydride was added
while the triethanolamine solution was being stirred. Slides were incubated for 10 min with continued stirring then rinsed in PBS for 2 min.

**Dehydration**

Slides were dehydrated in a graded ethanol series using the same ethanol series as before, but with fresh 100% ethanol for the final step. Slides were left to dry for 30 min or more.

**Hybridisation**

Working probe was made by diluting 1 µl of stock probe to 19 µl of 50% formamide per slide. The working probe was heated at 80°C for 2 min to relax secondary structures then cooled on ice before adding to the hybridisation buffer.

The following hybridisation buffer was used:
- 1 ml 10% (w/v) Boehringer blocking reagent (Roche)
- 1.125 ml 20xSSPE
- 1 g dextran sulfate
- 50 µl 100 mM DDT
- 100 µl 10 mg/ml tRNA
- 4 ml deionised formamide
- Water to 8 ml

20 µl of the working probe, 80 µl of hybridisation buffer and 1 µl of RNase OUT (Invitrogen) were used for each slide.

Hybridisation solution (100 µl) was pipetted onto each slide and covered with a glass coverslip, taking care to remove bubbles. Slides were transferred to a sealable plastic box lined with paper towels soaked in 50% (v/v) formamide. Sealed boxes were placed in a 50-55°C incubator and left to hybridise overnight.
Washes
The following morning the coverslips were removed, slides were returned to the rack and washed in 0.2% SSC for 1 h at 55°C and in fresh 0.2% SSC for a further hour. Slides were then immersed in 1xPBS for 10 min.

Blocks and antibody incubation
Blocking steps were carried out at room temperature. Slides were placed in plastic trays on a rocking platform and treated with a series of blocking solutions: Block A (1% Boehringer block in 1xTBS) for 45 min, Block A for 20 min, Block B (1% BSA, 0.3% Triton x-100 (Sigma) in 1xTBS) for 45 min.

Anti-DIG conjugated antibody (Roche) was diluted 1:1250 in Block B. 150 μl of antibody solution was pipetted onto each slide and slides were covered with glass coverslips. Slides were transferred to a sealable plastic box lined with damp paper towels, and incubated at room temperature for 2 h. Coverslips were removed and slides were returned to plastic trays. Slides were treated with four changes of Block B for 20 min each.

Slides were returned to the rack and immersed in Buffer C (100 mM Tris pH 9.5, 50 mM MgCl₂, 100 mM NaCl) for 15 min then in fresh Buffer C for a further 15 min.

Digoxigenin detection
100 μl of NBT/BCIP (Roche) was diluted in 5 ml of Buffer C just before use. 100 μl of solution was pipetted onto each slide and covered with a glass coverslip. Slides were transferred to a sealable plastic box lined with damp paper towels, and incubated at room temperature in complete darkness for 15 - 48 h. Staining intensity was checked under the stereomicroscope.

To stop the staining reaction, slides were returned to the rack and rinsed for 10 min in TE. Slides were allowed to air dry before mounting coverslips with immunomount (Shandon).
4.2.6 Husk leaf and prophyll material for *in situ* hybridisation

To determine the expression patterns of adaxially expressed genes in developing prophylls, husk leaves and vegetative leaves, tissue was fixed and probed for *zyb9* and *rld1* expression.

For husk leaves and prophylls, seedlings were harvested at 28 to 50 days after planting. Approximately seven outer leaves were removed from each, leaving lateral buds in upper nodes and their subtending leaves intact.

To facilitate comparisons of mutant and wild-type development, buds at similar developmental stages, and sections from equivalent positions within the bud were compared. Buds at similar developmental stages were identified by counting the number of leaves that had been initiated by the lateral meristem. Sections from equivalent positions were selected by counting the number of sections from the base of the bud.

For analysis of sheath margins, seedlings were harvested at 24-28 days after planting. The outer four leaves and the distal part of the shoot were removed.

Genetic material was chosen from backgrounds that exhibit the particular trait being studied. Husk leaf outgrowths and the subdivided keel phenotype were examined in *mwp1-R* introgressed into the W23 background, with wild-type W23 plants as a control. The unfused prophyll phenotype was studied in a non-inbred line that consistently showed this phenotype. Sheath margin outgrowths were examined in *mwp1-R* introgressed into the A188 background, with wild-type A188 as a control.

4.3 Results

4.3.1 Husk leaf phenotype

*mwp1-R* and wild-type husk leaves were examined by SEM and light microscopy of sectioned tissue (Figure 4.1, Figure 4.2). In transverse section the wild-type husk leaf has a ribbed appearance and the vascular bundles are oriented with xylem at the adaxial pole (false coloured pink) and phloem at the
abaxial pole (false coloured blue) (Figure 4.2 A, B). Wild-type husk leaves are relatively smooth on the adaxial surface, with brick-shaped cells and shorter prickle-like hairs (Figure 4.2 C). The abaxial surface is hairier, particularly near the margins, and the cells are more rounded (Figure 4.2 D).

Outgrowths on mwp1-R husk leaves generally occur in pairs on the abaxial surface (Figure 4.1 D, Figure 4.2 E, F). The outer surfaces of ectopic flaps are hairy and have rounded cells, similar to abaxial margin tissue (Figure 4.2 F). The epidermis between outgrowth pairs is smoother, with brick-like cells and only shorter prickle-like hairs, similar to adaxial epidermis (Figure 4.2 J). Vascular bundles are oriented with xylem to the inner side of ectopic flaps (Figure 4.2 G). Vascular bundles at junctions between outgrowths and the main lamina are often partially or fully radialised, with xylem surrounding phloem tissue (Figure 4.2 H, I).

Figure 4.1. Wild-type and mwp1-R ears. (A) Wild-type ear. (B) Abaxial surface of wild-type husk leaf. (C) mwp1-R ear. (D) Abaxial surface of mwp1-R husk leaf with ectopic outgrowths.
Figure 4.2. Ectopic outgrowths on mwp1-R husk leaves are associated with adaxialised tissue.

(A-D) Wild-type husk leaves have a polarised arrangement of vascular tissue and epidermal characteristics. (A) Transverse section of wild-type husk leaf. (B) The vascular bundles of wild-type husk leaves are oriented with xylem (false coloured pink) on the adaxial side and phloem (false coloured blue) on the abaxial side. (C) The adaxial epidermis is relatively smooth, has only shorter hairs, and the cells are brick-like. (D) The abaxial epidermis has a variety of hair types with long hairs near the margins, and more rounded cells.

(E-J) mwp1-R husk leaves develop pairs of ectopic outgrowths on the abaxial surface. (G) Vascular bundles are oriented with xylem to the inner side of tissue flaps. (H, I) Vascular bundles at outgrowth junctions are often partially radialised. (F) The outer epidermis of these outgrowths has long hairs characteristic of abaxial marginal tissue. (J) The epidermis between pairs of outgrowths is smooth with only shorter hairs and is more similar to adaxial epidermis. (J) is a piece of tissue adjacent to (F) that has been cut away to reveal the epidermis between tissue flaps. Red dotted lines indicate equivalent positions. Scale bars in (B), (H) and (I) = 20μm.
Expression of rld1 and zyb9 in husk leaves

To determine if genes that are normally expressed adaxially are misexpressed in mwp1-R husk leaves, developing husk leaves were probed with zyb9 and rld1 (Figure 4.3, Figure 4.4). Juarez et al. (2004b) found that zyb9 and zyb14 have similar expression patterns in vegetative leaf primordia, and my preliminary results indicated that they also have similar expression patterns in developing husk leaves. Therefore, it was decided to use zyb9 as a probe representative of the YABBY family. Transverse sections from the basal part of lateral buds are shown. In the centre of each is the ear axis (asterisks). Husk leaf primordia surround the ear axes.

rld1 is expressed on the adaxial side of wild-type husk leaf primordia (Figure 4.3 A-C). Ectopic outgrowths on mwp1-R husk leaves are associated with rld1 misexpression (Figure 4.3 D-F). Figure 4.3 D shows paired outgrowths on the abaxial side of husk leaves two and three (arrowheads). In the region between the outgrowths, rld1 is expressed both adaxially and abaxially. Expression is particularly strong on the abaxial side between outgrowths (Figure 4.3 E, F).

zyb9 is expressed on the adaxial side of young husk leaf primordia (data not shown). In older husk leaf primordia, zyb9 expression does not appear strongly polarised (Figure 4.4 A, B). zyb9 expression persists at the margins and in developing vascular bundles, but fades from medial regions.

Ectopic outgrowths on mwp1-R husk leaves are associated with zyb9 expression (Figure 4.4 C, D). Figure 4.4 C shows paired outgrowths on the abaxial side of husk leaves three and four (arrowheads). These outgrowths occur in the medial region of each husk leaf. zyb9 is expressed in each outgrowth (Figure 4.4 D), whereas zyb9 is not expressed in the medial region of wild-type husk leaves at similar stages (Figure 4.4 B).
Figure 4.3. *rld1* is misexpressed in *mwp1-R* husk leaf primordia. (A-C) *rld1* is expressed on the adaxial side of wild-type husk leaves. (A) Transverse section through wild-type lateral bud. The ear axis can be seen at the centre (asterisk), the culm is below and the subtending vegetative leaf is above. Developing husk leaves and the prophyll surround the ear axis. (B, C) Close-ups of wild-type husk leaves showing *rld1* expression on the adaxial side. Expression persists at the margins and in developing vascular bundles, but fades from the medial region.

(D-F) *rld1* expression in *mwp1-R* husk leaves. (D) Transverse section through *mwp1-R* lateral bud. Husk leaf primordia have ectopic outgrowths (arrowheads). *rld1* is expressed on the abaxial side, between pairs of ectopic outgrowths. (E, F) Close-ups of ectopic outgrowths in (D). Scale bars in (A) and (D) = 200μm. Scale bars in (B), (C), (E) and (F) = 50μm.
Figure 4.4. *zyb9* is misexpressed in *mwp1-R* husk leaf primordia. (A, B) *zyb9* expression in wild-type husk leaf primordia. (A) Transverse section through wild-type lateral bud. The ear axis can be seen at the centre (asterisk), the culm is below and the subtending vegetative leaf is above. Husk leaf primordia surround the ear axis. *zyb9* expression persists at the margins and in developing vascular bundles of older wild-type husk leaves. Expression fades from the medial region. (B) is a close-up of the medial region (arrowhead) of husk leaf three and margins (arrows) of husk leaf four.

(C, D) *zyb9* expression in *mwp1-R* husk leaf primordia. (C) Transverse section through *mwp1-R* lateral bud. Husk leaf primordia three and four have pairs of ectopic outgrowths on the abaxial side that express *zyb9* (arrowheads). (D) Close-up of ectopic outgrowths on husk leaf four. *zyb9* is expressed at the tip of each outgrowth. Arrowhead indicates medial region of husk leaf four, arrows indicate margins of husk leaf three. Note also that the prophyll is unfused and the keel on the right is subdivided into two points. Scale bars in (A) and (D) = 200µm. Scale bars in (B) and (D) = 50µm.
4.3.2 Prophyll phenotypes

The prophyll is the organ most severely affected by *mwp1-R*, although the phenotype varies in both penetrance and expression.

Wild-type prophylls

The prophyll is the first organ produced by a newly initiated lateral meristem (Arber, 1934). At maturity, the prophyll has two outer margins that enclose the ear, and two prominent keels that wrap around the culm (Figure 4.5, Figure 4.6 A-C) (Scanlon and Freeling, 1998). The two keel regions are connected by membranous tissue. Wild-type prophylls bifurcate at the very tip.

![Figure 4.5. The prophyll. In maize, the female inflorescences (ears) are produced by lateral meristems in the axils of vegetative leaves. The prophyll (blue) is the first organ initiated by the lateral meristem. At maturity, the prophyll is sandwiched between the ear and the culm (stem). Two prominent keels extend at right angles from the prophyll (arrowhead). The two outer margins enclose the ear, while the keels wrap around the culm.](image)

The adaxial epidermis of the wild-type prophyll is relatively smooth, with only shorter prickle-like hairs (Figure 4.6 H). The abaxial epidermis, including the keel region, is covered in longer hairs (Figure 4.6 I). Most veins are oriented with xylem at the adaxial pole and phloem at the abaxial pole (Figure 4.6 D, F, G). However, it was observed that individual veins in the keel often had a different orientation to veins in flanking regions (Figure 4.6 E, Figure 4.7). This
Figure 4.7. Vascular polarity in wild-type prophyll keel. (A) Transverse section through wild-type prophyll keel. (B-E) Close-ups of veins indicated by blue boxes in (A). (B, C) Veins in regions adjacent to the keel are oriented with xylem to the adaxial side and phloem to the abaxial side. (D) Partially radialised vascular bundle in keel. (E) Vein in keel with opposite orientation to veins adjacent to the keel. Sections are stained with safranin and fast green. Xylem elements stain red, whereas phloem cells stain green. (F) Schematic of vein orientation in keel region. Phloem is indicated by blue circles, xylem by red arrows. Scale bars in (B-E) = 20μm.
was observed in 4/5 examples in A188 prophylls and 4/4 times in W23 prophylls.

**mwp1-R unfused prophyll phenotype**

The prophyll is the organ most strongly affected by mwp1-R. In the most severe cases the prophyll is reduced to two unfused prongs (Figure 4.6 J-L). The central membrane and much of the outer margins are deleted. In addition, keel outgrowth is significantly reduced.

The adaxial epidermis of mwp1-R prophyll prongs appears normal (Figure 4.6 R). The abaxial side has normal looking epidermal features in the central part (indicated by yellow dotted line). However, the epidermis near the edges of the prong is smooth and more similar to adaxial epidermis (Figure 4.6 S). The orientation of vascular bundles within the prong varies. Bundles near the edge of the prong have a relatively normal orientation (Figure 4.6 M, N, Q), whereas bundles in more central regions may be completely or partially radially aligned (Figure 4.6 O, P). Within mwp1-R prophyll prongs, the distortions in vascular polarity are more extreme and affect a greater number of vascular bundles than the differently oriented vascular bundles seen in the keel region of wild-type prophylls.

**Comparison of mwp1-R and wild-type prophyll development**

To characterise normal prophyll development, and to determine when the mwp1-R unfused prophyll defect first becomes apparent, an investigation of prophyll development in mwp1-R and wild-type plants was undertaken by SEM (Figure 4.8). Leaf primordia are numbered in the order initiated, with primordia 1 and 2 comprising the prophyll and primordium 3 the first husk leaf. In wild-type, the first two primordia are connected by a strip of tissue at the time they emerge (Figure 4.8 A, B). At a similar stage, the two primordia comprising the mwp1-R prophyll also have a connecting membrane (Figure 4.8 E, F). By plastochron 4 (Figure 4.8 C), the fused region of wild-type prophylls has grown upward (along the proximal-distal axis) and the tips have grown laterally. The third primordium, the first husk leaf, can just be seen between the prophyll tips. In contrast, the connecting membrane of the mwp1-R prophyll has undergone little growth along the proximal-distal axis, and the tips have not grown laterally, leaving the
Figure 4.8. The *mwp1-R* prophyll defect is apparent by plastochron 4.
SEMs of wild-type (A-D) and *mwp1-R* (E-H) axillary buds with developing prophylls. At the earliest visible stages of prophyll development, the two primordia that comprise the prophyll are connected by a strip of tissue in both wild-type (A, B) and *mwp1-R* (E, F). By plastochron 4, the *mwp1-R* defect is apparent. In wild-type (C), leaves 1 and 2 have grown laterally and the central membrane has grown upwards, concealing the third primordium. In *mwp1-R* (G), leaves 1 and 2 have undergone little lateral growth and the central membrane has not elongated, leaving leaf 3 exposed. Later in development, these differences are even more pronounced (D, H). Early stage prophylls are shown from above (A, E) and from the side furthest from the culm (B, F). Later stages show the side adjacent to the culm (C, D, G, H). Leaves are numbered in the order of initiation. All scale bars = 100µm
third primordium exposed (Figure 4.8 G). At later stages this difference is even more apparent. In wild-type, the third primordium is enclosed by the prophyll (Figure 4.8 D), whereas in mwp1-R there is wide gap between the prongs that leaves the third primordium completely exposed (Figure 4.8 H).

**rld1 expression in early stage prophyll development**

*rld1* expression was analysed in early stage prophylls of wild-type and mwp1-R plants (Figure 4.9). Transverse sections of lateral buds are shown, with SEMs illustrating equivalent stages. In wild-type prophylls, *rld1* is expressed adaxially (Figure 4.9 D, E). Figure 4.9 D shows a young bud that is just initiating the third primordium (first husk leaf). Only one margin of the husk leaf can be seen in this section (arrow), as husk leaf primordia are initiated at an angle rather than perpendicular to the main shoot axis. In very early stage prophylls, a block of expression is often seen at the outer edge, such that a boundary of *rld1* expression extends along the keel axis (Figure 4.9 D, Figure 4.10). This block of expression is not seen at later stages, when expression becomes confined to the adaxial side of the prophyll (Figure 4.9 E). Expression persists at the outer margins and on the adaxial side of the central membrane, but often fades in the intervening region.

*rld1* expression was analysed in mwp1-R prophylls, in a genetic background that consistently exhibits the unfused prophyll phenotype. Unlike wild-type prophylls, *rld1* expression is seen on the abaxial side of the mwp1-R prophyll very early in development (arrowhead in Figure 4.9 I). One margin of the third primordium (first husk leaf) can be seen (arrow). Figure 4.9 J shows a bud at a later stage, equivalent to the wild-type bud shown in Figure 4.9 E. The prophyll is clearly unfused and the two prongs show patchy *rld1* expression throughout. The husk leaves display a normal-looking adaxial expression pattern.
Figure 4.9. The mwp1-R unfused prophyll phenotype is associated with rld1 misexpression early in development.

SEMs of lateral buds showing early stages of prophyll development in wild-type (A-C) and in situ hybridisation of rld1 in transverse sections of equivalent stage buds (D, E). rld1 is normally expressed on the adaxial side of developing prophylls and husk leaves (D, E). Blocks of rld1 expression can be seen at the prophyll outer margins early in development (D). These fade at later stages (E).

SEMs (F-H) and in situ hybridisation of rld1 (I, J) in mwp1-R lateral buds. In mwp1-R, rld1 expression is seen on the abaxial side of the prophyll early in development (arrowhead in I). Later, diffuse rld1 expression is seen throughout the two prongs of the unfused prophyll primordium (J). Prophyll primordia are outlined with dotted lines in (D), (E), (I) and (J). Arrows in (D) and (I) indicate P3 leaf primordia. Scale bars = 100μm.
Figure 4.10. *rld1* expression in wild-type prophyll primordium. *rld1* is expressed on the adaxial side of the prophyll primordium (outlined), and in blocks at the outer margins. Boundaries of expression extend along the keel axes (red dotted lines). Two sets of margins are indicated by green arrows. *rld1* expression persists at the margins and fades from the intervening regions. Green arrowhead represents the point at which the two primordia fuse according to the fused phytomer model. OM=outer margins, IM=inner margins. LM = lateral meristem. P3 = plastochron 3 leaf primordium, the first husk leaf to be initiated after the prophyll.

**Morphometric analysis of *mwp1*-R and wild-type prophylls**

To investigate prophyll growth further, the length of the fused region was compared in *mwp1*-R and normal prophylls at different stages of development. The length of the fused region was plotted against overall prophyll length (Figure 4.11). At the earliest stages (up to about 1 mm overall length) the length of the fused region appears similar in *mwp1*-R and normal prophylls. For prophylls over 1 mm in length, there is a clear difference between the length of the fused region in *mwp1*-R and wild-type. This difference becomes increasingly apparent as growth proceeds. In wild-type the fused region appears to grow rapidly, whereas in *mwp1*-R this region grows very slowly. By the time the prophylls were 10 mm in height, all wild-type prophylls had a fused region of 4 mm or longer, whereas none of the *mwp1*-R fused regions had reached 2 mm.

**mwp1*-R unfused prophylls with tabs**

A variation of the unfused prophyll phenotype is the development of prophylls that are unfused, but have a "tab" of tissue extending from the membranous strip that links the two prongs (Figure 4.12). On either side of the tab are U-shaped sinuses that never elongate. The tabs vary in size and distribution of
Figure 4.11. Morphometric analysis of mwp1-R and wild-type prophyll development. 
Prophyll length and the length of the fused region were measured in developing wild-type and 
mwp1-R prophylls (illustrated in A and B). The length of the fused region was plotted against 
overall prophyll length (C). (D) is an enlargement of the boxed portion in (C). Scale bars in (A) 
and (B) = 500 μm.
adaxial and abaxial cell types. Figure 4.12 (D-F) shows a narrow tab that has relatively normal adaxial (E) and abaxial (F) cell types. Figure 4.12 (G-I) shows a wider tab. The adaxial surface has normal looking epidermal characteristics while the abaxial surface has a series of outgrowths. The polarity of veins within prophyll tabs varied from relatively normal to partially radially, with xylem surrounding phloem.

Figure 4.12. The *mwp1-R* prophyll “tab” phenotype. Photographs and SEMs of wild-type prophyll (A-C) and *mwp1-R* prophylls with tabs (D-I). (A) The wild-type prophyll is fused. (B) The adaxial surface of the central membrane is relatively smooth with prickle-type hairs. (C) Cells on the abaxial surface are more rounded. (D) *mwp1-R* prophyll with narrow tab. The adaxial (E) and abaxial (F) epidermis of the tab appear relatively normal. (G) *mwp1-R* prophyll with wider tab. (H) The adaxial epidermis of the tab appears normal. (I) The abaxial side of the tab has ectopic outgrowths. Scale bars = 100μm.
**rld1 expression in mwp1-R prophylls with developing tabs**

Expression of *rld1* was examined in a *mwp1-R* prophyll with a developing tab, and in a wild-type prophyll at an equivalent stage (Figure 4.13). The wild-type prophyll is fused, and *rld1* expression is adaxial (Figure 4.13 A, B). The SEM shows a prophyll at a slightly later stage than the section. The dotted line indicates the position equivalent to the plane of the section.

The SEM in Figure 4.13 C shows a *mwp1-R* prophyll with a developing tab. Figure 4.13 D-F are serial sections of a *mwp1-R* prophyll at a slightly earlier stage. Sections are shown corresponding to the fused region at the base of the prophyll (E, F) and a more distal section where the tab and two prophyll prongs are separate (D). In D, an island of tissue corresponding to the tab can be seen (asterisk). Dotted lined in Figure 4.13 C indicate positions equivalent to where sections were taken. In the proximal sections (Figure 4.13 E, F), adaxial *rld1* expression is seen in two patches just below the U-shaped sinuses (arrowheads). The region immediately below the tab retains a more polar pattern of expression, although expression is somewhat patchy (arrows).

**mwp1-R subdivided keel phenotype**

In the W23 background, *mwp1-R* prophylls were generally fused. However, in many cases each of the keels was replaced by a complex series of outgrowths (Figure 4.14 A-C). Vascular bundles in the keel region were partially or completely radialised, with xylem surrounding a central strand of phloem tissue (Figure 4.14 E-G). Vascular bundles in regions adjacent to the keel had a more normal orientation (Figure 4.14 D).

*rld1* and *zyb9* expression patterns were examined in developing prophylls of W23 plants and *mwp1-R* introgressed into W23. In wild-type W23 plants, *rld1* is expressed on the adaxial side of the prophyll and expression persists at the margins and in developing vascular bundles. *rld1* is not expressed in the keels of later stage W23 prophylls (Figure 4.14 H). In the *mwp1-R* prophyll shown in Figure 4.14 I, the keels appear blunter than normal and the keel on the left is divided into two points. *rld1* expression is similar to wild-type at the margins and in developing vascular bundles (Figure 4.14 I). However, *rld1* expression is seen in the keel, particularly in patches where outgrowths are developing.
Figure 4.13. \textit{rld1} expression in \textit{mwp1-R} prophyll exhibiting the “tab” phenotype. (A) SEM of wild-type prophyll. (B) \textit{In situ} hybridisation of \textit{rld1} in wild-type prophyll at slightly earlier stage than (A). Dotted line in (A) indicates approximate position of section shown in (B). \textit{rld1} expression is confined to the adaxial side of the prophyll. (C) SEM of \textit{mwp1-R} prophyll with developing tab. (D-F) \textit{In situ} hybridisation of \textit{rld1} in \textit{mwp1-R} prophyll at slightly earlier stage than (C). Dotted lines in (C) indicate approximate position of sections in (D-F). (D) Section taken proximal to fused region. The tab appears as an island of tissue separate from the two prophyll prongs (asterisk). (E,F) Sections through the fused region. \textit{rld1} is expressed on both the adaxial and abaxial sides in the regions immediately below the sinuses (arrowheads). \textit{rld1} expression is largely confined to the adaxial domain in the region immediately below the tab (arrows). Scale bars in (A) and (C) = 200\mu m, Scale bars in (B) and (D-F) = 100\mu m.
Figure 4.14. mwp1-R subdivided keel phenotype. (A) mwp1-R ear with prophyll exhibiting the subdivided keel phenotype. The prophyll is narrow and twists away from the ear. Transverse section (B) and SEM (C) of mwp1-R keel. The keel is reduced to a complex series of outgrowths. The prophyll outer margin (om) and central membrane (cm) continue out of the plane of the photograph (B). (D-G) Enlargements of veins shown in (B). Veins in the keel region (E-G) are radialised, with xylem (false coloured pink) surrounding phloem. (H) In situ hybridisation of rld1 in transverse section of wild-type lateral bud. Prophyll is outlined with dashed line. rld1 is not expressed in the prophyll keels (arrowheads). (I) In situ hybridisation of rld1 in mwp1-R lateral bud. Patches of rld1 expression are seen in the prophyll keels (arrowheads). The keels are blunter, and the keel on the left is subdivided into two points. Scale bars in (D-G) = 50μm.
zyb9 and rld1 have similar expression patterns in wild-type prophyll primordia and zyb9 is misexpressed in mwp1-R prophyll primordia in a pattern similar to rld1 misexpression (data not shown).

### 4.3.3 mwp1-R sheath margin phenotype

The sheaths of mwp1-R vegetative leaves develop pairs of outgrowths similar to those seen on mwp1-R husk leaves, but these are generally less prominent. Pronounced single outgrowths often occur at the sheath margins (Figure 4.15 D, E). The true margins of affected sheaths are blunt compared to wild-type sheath margins which are normally tapered (arrows Figure 4.15 A, E). Outgrowths occur on the abaxial side, adjacent to the true margin (Figure 4.15 E). These outgrowths are tapered and look more like normal margins. The epidermis on the outer side of sheath outgrowths is smooth and similar to normal adaxial epidermis (Figure 4.16 A, E), whereas the epidermis on the side nearer the midrib is hairy like abaxial epidermis (Figure 4.16 B, F). The vascular bundles near the true margin of the mwp1-R leaf have xylem on both sides, with phloem at the centre (Figure 4.15 F, G). Cells on the marginal side of mwp1-R sheath outgrowths were shorter than wild-type adaxial sheath margin cells (Figure 4.16 D, E).

rld1 expression was examined in wild-type leaf primordia and mwp1-R leaf primordia with developing sheath margin outgrowths. rld1 is normally expressed on the adaxial side of young leaf primordia. Expression persists at the margins and in developing vascular bundles of older leaf primordia (Figure 4.15 C, I, J; (Juarez et al., 2004b). In the mwp1-R leaf shown, rld1 expression is seen throughout the sheath margin (Figure 4.15 I, J). An outgrowth is developing along the boundary of rld1-expressing and non-expressing tissue. The other sheath margin shows a normal rld1 expression pattern and is developing normally.
Figure 4.15. Outgrowths at mwp1-R sheath margins are associated with ectopic rld1 expression.
In wild-type leaves, the sheath margin is tapered (arrow in A) and vascular bundles are oriented with xylem on the adaxial side and phloem on the abaxial side (B). (C) In situ hybridisation in wild-type leaf primordia shows rld1 is expressed on the adaxial side of developing leaves and persists at the margins mwp1-R leaves often develop ectopic outgrowths at the sheath margins (D, E). The true sheath margin is blunt (arrow), whereas the ectopic outgrowth is tapered and more similar to a normal sheath margin. Vascular bundles are often radial or mis-oriented (F, G, H). (I) and (J) show a mwp1-R sheath margin with an emerging outgrowth. rld1 is expressed throughout the true margin and the outgrowth coincides with the boundary of rld1 expression. The other margin (below) expresses rld1 on the adaxial side and is developing normally. Sections shown in (A), (B) and (E-H) are stained with safranin and fast green. Xylem elements stain red, whereas phloem cells stain green. Scale bars in (A, C, E, J) = 200μm. Scale bars in (B and F-H)=50μm.
Figure 4.16. Adaxial epidermal characteristics continue onto the abaxial side of *mwp1-R* sheath margins with ectopic outgrowths. (A) The adaxial epidermis of wild-type sheath margin is smooth with long, brick-like cells. (B) The abaxial epidermis is hairy and the cells are more rounded. (C) Handsection of *mwp1-R* sheath margin with ectopic outgrowth. (D) The adaxial epidermis of the *mwp1-R* sheath margin is smooth like wild-type adaxial epidermis, but the cells appear shorter. (E) The side of the outgrowth nearest the margin is smooth with brick-like cells, similar to normal adaxial epidermis, although the cells appear shorter. (F) The epidermis on the other side of the eptopic outgrowth has the features of normal abaxial epidermis. Yellow dotted line in (C) indicates the extent of adaxial epidermal characteristics, which appear to wrap around the true margin. Blue dotted line indicates abaxial epidermal characteristics. Scale bars = 100μm.
4.3.4 mwp1-R floral organ phenotypes

Floral organs such as glumes, paleae and carpels are homologous to leaves. Mutations that disrupt leaf development may affect other lateral organs in a similar manner (Bossinger et al., 1992; Scanlon and Freeling, 1998). Therefore, mwp1-R floral organs were examined to determine if they exhibit a phenotype.

Glumes and paleae

The mwp1-R glumes and paleae that were examined by SEM did not have any obvious outgrowths or ectopic tissues. When entire male spikelets were examined by SEM, the florets were visible in mwp1-R spikelets but not in wild-type spikelets. This suggested that mwp1-R glumes may be narrower than wild-type glumes.

Silks

Wild-type (A188) silks are long and straight, with regular files of cells and hairs on the outer edges (Figure 4.17 A, B). Wild-type silks are oval in transverse section, with veins at either end and an indented region (arrow) between the veins (Figure 4.17 C).

In the A188 background, mwp1-R silks frequently had a kinked or twisted appearance – this was particularly pronounced near the base of the silk (Figure 4.17 D, E). Extensive folds of tissue occur in the indented region (arrow Figure 4.17 E). In addition, mwp1-R silks often had less pronounced outgrowths along their outer edges. In transverse section, the shape of mwp1-R silks is less regular and the veins appear closer together than in wild-type silks (Figure 4.17 F). Epidermal cells appear larger and are oriented differently, with some elongated perpendicular to the epidermis.

mwp1-R silks in the W23 background also showed some twisting, but this was less pronounced than in the A188 background.
Wild-type milkweed pod1-R

Figure 4.17. *mwp1-R* silks are twisted and have ectopic outgrowths. (A, B) Wild-type silks are straight, with regular files of cells and hairs along the outer edges. (C) Wild-type silks are oval in transverse section, with veins at either edge. Arrows in (B) and (C) indicate indented region. (D, E) The *mwp1-R* silk is twisted, with ectopic outgrowths in the indented region (arrow). Less pronounced outgrowths occur along the outer edge. (F) The *mwp1-R* silk is narrower and less regular in shape. Epidermal cells are larger than in wild-type and some are elongated perpendicular to the surface of the silk. Scale bars = 200μm.
4.3.5 Measurements of mature wild-type and mwp1-R lateral organs

To investigate the effects of mwp1-R on lateral organ growth, mature leaves and floral organs were measured. The mean dimensions of mwp1-R and wild-type lateral organs were compared. The plant material used for these measurements was wild-type W23 and A188 and mwp1-R introgressed into the W23 and A188 backgrounds.

Prophylls

In the W23 background, mwp1-R prophylls were significantly narrower than wild-type prophylls (Table 4.1 A). The distance between the keels was also significantly narrower in mwp1-R. On average, wild-type prophylls were 43.6 mm wide and mwp1-R prophylls were 20.8 mm wide. The average distance between the keels was 16.4 mm in W23 and 7.5 mm in mwp1-R. Thus, mwp1-R prophylls were 48% as wide as wild-type prophylls, and the distance between the keels was 46% as wide. There was no significant difference in prophyll length between mwp1-R and wild-type prophylls in the W23 background.

In the A188 background, mwp1-R prophylls were narrower than wild-type prophylls, although the difference was not as great as in the W23 background (Table 4.1 B). mwp1-R prophylls were also significantly shorter than wild-type prophylls in this background. On average, wild-type prophylls were 38.2 mm wide and mwp1-R prophylls were 24.2 mm wide. The average distance between the keels was 12.4 mm in A188 and 7.6 mm in mwp1-R. The average prophyll length was 181.5 mm in wild-type and 120.7 mm in mwp1-R. Thus, mwp1-R prophylls were 67% as wide as wild-type prophylls, the distance between the keels was 63% as wide, and mwp1-R prophylls were 67% as long as wild-type prophylls.
Table 4.1. Measurements of mature mwp1-R and wild-type prophylls. (A) W23 background. (B) A188 background. Prophyll length was measured from base to tip. Prophyll width and the distance between the keels were measured at a point midway between the base and tip of the prophyll. Mean values were calculated separately for mwp1-R and wild-type prophylls. Means were compared by Student’s t-test to determine if they are significantly different at the 0.05 confidence level. * indicates values that are significantly different. SE = standard error. Numbers in brackets are the mwp1-R value represented as a percentage of the wild-type value.

A

<table>
<thead>
<tr>
<th>Prophyll W23 background</th>
<th>Wild-type (n=13)</th>
<th>mwp1-R (n=15)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>Mean SE</td>
<td>154.6 5.5</td>
<td>148.5 6.3</td>
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<tr>
<td>Width (mm)</td>
<td>Mean SE</td>
<td>43.6 2.4</td>
<td>20.8 (48%) 2.1</td>
</tr>
<tr>
<td>Between keels (mm)</td>
<td>Mean SE</td>
<td>16.4 0.8</td>
<td>7.5 (46%) 0.8</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Prophyll A188 background</th>
<th>Wild-type (n=13)</th>
<th>mwp1-R (n=13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>Mean SE</td>
<td>181.5 4.6</td>
<td>120.7 (67%) 3.8</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>Mean SE</td>
<td>38.2 1.1</td>
<td>24.2 (63%) 1.6</td>
</tr>
<tr>
<td>Between keels (mm)</td>
<td>Mean SE</td>
<td>12.4 0.2</td>
<td>7.6 (61%) 0.4</td>
</tr>
</tbody>
</table>

Husk leaves

In the W23 background, mwp1-R husk leaves were significantly narrower than wild-type husk leaves (Table 4.2 A). Husk leaf length was not significantly different. Husk leaves 1, 4 and 8 (with 1 being the outermost husk leaf) were measured. The average width of the first husk leaf was 89.9 mm in wild-type plants and 53.1 mm in mwp1-R. The average width of husk leaf 4 was 108.3 mm in wild-type plants and 83.6 mm in mwp1-R. The average width of husk leaf 8 was 83.2 mm in wild-type plants and 55.2 mm in mwp1-R.

In the A188 background, husk leaves 1, 4 and 8 were all significantly narrower and shorter than equivalent wild-type husk leaves (Table 4.2 B). The average width of husk leaf 1 was 85.2 mm in wild-type plants and 51.3 mm in mwp1-R. The average length was 189.5 mm in wild-type plants and 171.4 mm in mwp1-R. The average width of husk leaf 4 was 98.3 mm in wild-type plants and 79.9 mm in mwp1-R. The average length was 194.2 mm in wild-type plants and 168.9 mm in mwp1-R. The average width of husk leaf 8 was 63.5 mm in wild-
type plants and 53.2 mm in \textit{mwp1-R}. The average length was 181.8 mm in wild-type plants and 155.8 mm in \textit{mwp1-R}.

Table 4.2. Measurements of mature wild-type and \textit{mwp1-R} husk leaves. (A) W23 background. (B) A188 background. Husk leaves one, four and eight were measured, with one being the outermost husk leaf. Husk leaf length was measured from the base to the blade-sheath boundary. Width was measured at a point midway between the base and tip of the leaf. Mean values were calculated separately for wild-type and \textit{mwp1-R} husk leaves. Means were compared by Student’s t-test to determine if they are significantly different at the 0.05 confidence level. * indicates values that are significantly different. SE = standard error. Numbers in brackets are the \textit{mwp1-R} value represented as a percentage of the wild-type value.

<table>
<thead>
<tr>
<th>Husk leaf 1</th>
<th>Wild-type</th>
<th>\textit{mwp1-R}</th>
<th>P-value</th>
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<tbody>
<tr>
<td>W23 background</td>
<td>Mean (n=11)</td>
<td>Mean (n=15)</td>
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<tr>
<td>Length (mm)</td>
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<td>187.2</td>
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<tr>
<td>Width (mm)</td>
<td>89.9</td>
<td>53.1 (59%)</td>
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<table>
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<th>\textit{mwp1-R}</th>
<th>P-value</th>
</tr>
</thead>
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<td>W23 background</td>
<td>Mean (n=11)</td>
<td>Mean (n=15)</td>
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<tr>
<td>Length (mm)</td>
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<td>173.7</td>
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<tr>
<td>Width (mm)</td>
<td>108.3</td>
<td>83.6 (77%)</td>
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<th>P-value</th>
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<tr>
<td>Length (mm)</td>
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<tr>
<td>Width (mm)</td>
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<td>55.2 (66%)</td>
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<table>
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<tr>
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<th>P-value</th>
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<td>Mean (n=13)</td>
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<tr>
<td>Length (mm)</td>
<td>189.5</td>
<td>171.4 (90%)</td>
<td>0.013*</td>
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<tr>
<td>Width (mm)</td>
<td>85.2</td>
<td>51.3 (60%)</td>
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<td>A188 background</td>
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<tr>
<td>Length (mm)</td>
<td>194.2</td>
<td>168.9 (87%)</td>
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<tr>
<td>Width (mm)</td>
<td>98.3</td>
<td>79.9 (81%)</td>
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<th>Husk leaf 8</th>
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<td>Mean (n=13)</td>
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<tr>
<td>Length (mm)</td>
<td>181.8</td>
<td>155.8 (86%)</td>
<td>0.0097*</td>
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<tr>
<td>Width (mm)</td>
<td>63.5</td>
<td>53.2 (84%)</td>
<td>0.037*</td>
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In both backgrounds, prophyll width was more severely affected than later husk leaves. \textit{mwp1-R} had a more severe effect on prophyll and husk leaf width in the W23 background than in A188, but did not significantly affect husk leaf length in the W23 background. In the A188 background, \textit{mwp1-R} affected both the width and length of prophylls and husk leaves.

**Vegetative leaves**

Vegetative leaf size was not affected by \textit{mwp1-R} in either the W23 or A188 backgrounds. No significant differences were seen in blade length, sheath length, blade width or sheath width in \textit{mwp1-R} leaves compared to wild-type leaves (Table 4.3 A, B).

**Table 4.3. Measurements of mature wild-type and \textit{mwp1-R} vegetative leaves.** (A) W23 background. (B) A188 background. Leaves eight, nine and ten were measured (counting down from the tassel). Blade length was measured from the blade-sheath boundary to the tip of the leaf. Sheath length was measured from the base of the leaf to the blade-sheath boundary. Half leaf widths were measured from midrib to margin in the blade and sheath. Blade width was measured at the base of the blade. Sheath width was measured at a point midway between the base of the leaf and the blade-sheath boundary. Mean values were calculated separately for wild-type and \textit{mwp1-R} husk leaves. Means were compared by Student’s t-test to determine if they are significantly different at the 0.05 confidence level. SE = standard error.

<table>
<thead>
<tr>
<th>Vegetative leaf 8 A188 background</th>
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<tbody>
<tr>
<td>Blade length (mm)</td>
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<td>662.5 11.4</td>
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<td>Sheath length (mm)</td>
<td>Mean 130.3 3.1</td>
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<tr>
<td>Blade width (mm)</td>
<td>Mean 35.4 2.7</td>
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<tr>
<td>Sheath width (mm)</td>
<td>Mean 40.8 1.9</td>
<td>37.1 1.3</td>
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<table>
<thead>
<tr>
<th>Vegetative leaf 9 A188 background</th>
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<th>\textit{mwp1-R} (n=10)</th>
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<tr>
<td>Blade length (mm)</td>
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<td>Sheath length (mm)</td>
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<td>132.4 5.7</td>
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<tr>
<td>Blade width (mm)</td>
<td>Mean 32.5 2.9</td>
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<td>Mean 36.8 1.7</td>
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B

<table>
<thead>
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<th>Vegetative leaf 8 W23 background</th>
<th>Wild-type (n=10)</th>
<th>mwp1-R (n=11)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blade length (mm)</td>
<td>Mean</td>
<td>569.7</td>
<td>605.2</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>18.4</td>
<td>11.5</td>
</tr>
<tr>
<td>Sheath length (mm)</td>
<td>Mean</td>
<td>138.2</td>
<td>137.2</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>5.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Blade width (mm)</td>
<td>Mean</td>
<td>30.2</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Sheath width (mm)</td>
<td>Mean</td>
<td>33.3</td>
<td>35.5</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.7</td>
<td>1.0</td>
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</table>

<table>
<thead>
<tr>
<th>Vegetative leaf 9 W23 background</th>
<th>Wild-type (n=10)</th>
<th>mwp1-R (n=10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blade length (mm)</td>
<td>Mean</td>
<td>613.8</td>
<td>602.7</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>18.3</td>
<td>19.3</td>
</tr>
<tr>
<td>Sheath length (mm)</td>
<td>Mean</td>
<td>143.9</td>
<td>147.4</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>4.4</td>
<td>4.1</td>
</tr>
<tr>
<td>Blade width (mm)</td>
<td>Mean</td>
<td>24.6</td>
<td>28.1</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>2.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Sheath width (mm)</td>
<td>Mean</td>
<td>32.8</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vegetative leaf 10 W23 background</th>
<th>Wild-type (n=10)</th>
<th>mwp1-R (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blade length (mm)</td>
<td>Mean</td>
<td>626.1</td>
<td>616.5</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>20.6</td>
<td>23.0</td>
</tr>
<tr>
<td>Sheath length (mm)</td>
<td>Mean</td>
<td>149.1</td>
<td>154.0</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>4.5</td>
<td>4.6</td>
</tr>
<tr>
<td>Blade width (mm)</td>
<td>Mean</td>
<td>27.2</td>
<td>27.1</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Sheath width (mm)</td>
<td>Mean</td>
<td>32.7</td>
<td>33.6</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>
Glumes

In the W23 background, *mwp1-R* glumes were significantly narrower than wild-type glumes (Table 4.4 A). The average glume width was 4.1 mm in wild-type plants and 3.5 mm in *mwp1-R* plants. Thus, *mwp1-R* glumes were 85% as wide as wild-type glumes. The length of *mwp1-R* glumes was not significantly different from wild-type glumes in the W23 background.

In the A188 background, *mwp1-R* glumes were significantly narrower and shorter than wild-type glumes (Table 4.4 B). The average glume width was 3.9 mm in wild-type and 3.3 mm in *mwp1-R*. Thus, *mwp1-R* glumes were 83% as wide as wild-type glumes. The average glume length was 9.6 mm in wild-type and 8.9 mm in *mwp1-R*. Thus, *mwp1-R* glumes were 92% as wide as wild-type glumes in the A188 background.

Table 4.4. Measurements of mature wild-type and *mwp1-R* glumes. (A) W23 background. (B) A188 background. Length was measured from the base to the tip of the glume. Width was measured from margin to margin at the widest point. Mean values were calculated separately for wild-type and *mwp1-R* glumes. Means were compared by Student’s t-test to determine if they are significantly different at the 0.05 confidence level. * indicates values that are significantly different. SE = standard error. Numbers in brackets are the *mwp1-R* value represented as a percentage of the wild-type value.

<table>
<thead>
<tr>
<th>Glumes W23 background</th>
<th>Wild-type (n=20)</th>
<th><em>mwp1-R</em> (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>Mean 8.1 0.1</td>
<td>8.1 0.2</td>
<td>0.810</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>Mean 4.1 0.1</td>
<td>3.5 (85%) 0.1</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glumes A188 background</th>
<th>Wild-type (n=15)</th>
<th><em>mwp1-R</em> (n=15)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>Mean 9.6 0.2</td>
<td>8.9 (92%) 0.3</td>
<td>0.039*</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>Mean 3.9 0.1</td>
<td>3.3 (83%) 0.2</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

Paleae

In the W23 background, *mwp1-R* paleae were significantly narrower than wild-type paleae (Table 4.5 A). The average palea width was 3.8 mm in wild-type and 3.5 mm in *mwp1-R*. Thus, *mwp1-R* paleae were 92% as wide as wild-type
paleae. The length of \textit{mwp1-R} paleae and distance between the keels were not significantly different from wild-type in the W23 background.

In the A188 background, the length and width of \textit{mwp1-R} paleae was not significantly different from wild-type paleae (Table 4.5 B). The distance between the keels of \textit{mwp1-R} paleae was significantly narrower than in wild-type paleae.

Table 4.5. Measurements of mature wild-type and \textit{mwp1-R} paleae. (A) W23 background. (B) A188 background. Length was measured from the base to the tip of the palea. Palea width and the distance between the keels were measured at the widest point. Mean values were calculated separately for wild-type and \textit{mwp1-R} paleae. Means were compared by Student’s t-test to determine if they are significantly different at the 0.05 confidence level. * indicates values that are significantly different. SE = standard error. Numbers in brackets are the \textit{mwp1-R} value represented as a percentage of the wild-type value.

<table>
<thead>
<tr>
<th>Palea W23 background</th>
<th>Wild-type (n=25)</th>
<th>\textit{mwp1-R} (n=25)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>Mean 7.9 0.1</td>
<td>7.7 0.1</td>
<td>0.325</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>Mean 3.8 &lt;0.1</td>
<td>3.5 (92%) 0.1</td>
<td>0.010*</td>
</tr>
<tr>
<td>Between keels (mm)</td>
<td>Mean 1.3 &lt;0.1</td>
<td>1.2 0.1</td>
<td>0.613</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Palea A188 background</th>
<th>Wild-type (n=20)</th>
<th>\textit{mwp1-R} (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>Mean 8.2 0.1</td>
<td>8.2 0.1</td>
<td>0.880</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>Mean 3.8 0.1</td>
<td>3.5 0.1</td>
<td>0.073</td>
</tr>
<tr>
<td>Between keels (mm)</td>
<td>Mean 1.7 &lt;0.1</td>
<td>1.4 0.1</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

**Silks**

In the W23 background, the distance between the veins of \textit{mwp1-R} silks was significantly narrower than in wild-type silks (Table 4.6 A). This distance was 315 \textmu m in wild-type and 282 \textmu m in \textit{mwp1-R}. Thus, the intervein distance was 89\% as wide in \textit{mwp1-R} compared to wild-type. The distance from the vein to the outer edge of the silk was greater in \textit{mwp1-R} than in wild-type silks, measuring 65 \textmu m in wild-type and 84 \textmu m in \textit{mwp1-R}.
In the A188 background, the distance between the veins of mwp1-R silks was significantly narrower than in wild-type silks (Table 4.6 B). This distance was 352 μm in wild-type and 255 μm in mwp1-R. Thus, the intervein distance was 73% as wide in mwp1-R compared to wild-type. The distance from the vein to the outer edge of the silk was greater in mwp1-R than in wild-type silks, measuring 77 μm in wild-type and 105 μm in mwp1-R.

Table 4.6. Measurements of wild-type and mwp1-R silks. (A) W23 background. (B) A188 background. Measurements were made from transverse sections of wild-type and mwp1-R silks. Measurements were made of the distance between the veins, and from the vein to the outer edge of the silk. Mean values were calculated separately for wild-type and mwp1-R silks. Means were compared by Student’s t-test to determine if they are significantly different at the 0.05 confidence level. * indicates values that are significantly different. SE = standard error. Numbers in brackets are the mwp1-R value represented as a percentage of the wild-type value.

<table>
<thead>
<tr>
<th>Silk width</th>
<th>W23 background</th>
<th>Wild-type (n=8)</th>
<th>mwp1-R (n=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interven distance (μm)</td>
<td>Mean</td>
<td>315</td>
<td>282 (89%)</td>
<td>0.018*</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>8.1</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>Vein to outer edge (μm)</td>
<td>Mean</td>
<td>65</td>
<td>84 (129%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.4</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Silk width</th>
<th>A188 background</th>
<th>Wild-type (n=8)</th>
<th>mwp1-R (n=10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interven distance (μm)</td>
<td>Mean</td>
<td>352</td>
<td>255 (73%)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>11.7</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Vein to outer edge (μm)</td>
<td>Mean</td>
<td>77</td>
<td>105 (137%)</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>4.2</td>
<td>5.4</td>
<td></td>
</tr>
</tbody>
</table>
4.3.6 The mwp1-R;Wab1-R leaf blade phenotype

To determine if mwp1-R affects the polarity of ectopic sheath tissue, mwp1-R was crossed to Wab1-R, Kn1-R and Rs1-R. In mwp1-R vegetative leaves, disruption of adaxial-abaxial polarity is generally confined to the sheath. However, in mwp1-R;Wab1-R double mutants, adaxial-abaxial polarity is disrupted in blade tissue adjacent to ectopic sheath-like tissue (Figure 4.18). Bulliform cells and macrohairs are normally found only on the adaxial surface of the blade (Figure 4.18 B, C). In mwp1-R;Wab1-R mutants, bulliform cells and macrohairs are seen on both the adaxial and abaxial surfaces of the blade in regions near ectopic sheath-like tissue (D, E). Small outgrowths of blade tissue occur immediately adjacent to ectopic sheath-like tissue (D, E). Vascular polarity in these regions is disrupted in a similar manner to veins associated with outgrowths on mwp1-R husk leaves. Veins within outgrowths are oriented with xylem toward the inner surface of ectopic outgrowths (D) and in some cases are completely radialised (F). It was not clear whether adaxial-abaxial polarity was disrupted within the ectopic sheath-like tissue itself, as clearings tend to be narrow with few veins. Epidermal characteristics of cleared regions appeared to have normal adaxial-abaxial polarity, with smoother epidermis on the adaxial side and epidermal hairs on the abaxial side.

Similar disruptions to blade adaxial-abaxial polarity were associated with ectopic sheath-like tissue in mwp1-R;Kn1-R and mwp1-R;Rs1-R double mutants (data not shown).
Figure 4.18. Adaxial-abaxial polarity is disrupted in regions adjacent to ectopic sheath-like tissue in mwp1-R;Wab1-R leaf blades.

(A) mwp1-R;Wab1-R leaf blade with ectopic sheath-like tissue (arrowhead). (B) SEM of wild-type blade adaxial surface. (C) Transverse section of wild-type leaf blade. Normal blade tissue has macrohairs and bulliform cells on the adaxial epidermis (B, arrow in C). Vascular bundles are oriented with xylem on the adaxial side (false coloured pink) and phloem on the abaxial side (false coloured blue). (D) Transverse section of mwp1-R;Wab1-R leaf blade. (E) SEM of mwp1-R Wab1-R leaf blade abaxial surface. Narrow strips of ectopic sheath-like tissue are indicated by blue bars in (D) and (E). mwp1-R;Wab1-R leaf blades develop outgrowths adjacent to ectopic sheath-like tissue (D,E). Adjacent blade tissue has macrohairs (arrows) and bulliform cells (arrowheads) on the abaxial surface. Vascular polarity is distorted in ectopic outgrowths, and in some cases veins are completely radialised (D,E).
4.4 Discussion

Characterisation of the mwp1-R phenotype revealed that mwp1-R affects a range of lateral organs, including vegetative leaves, husk leaves, prophylls and floral organs. mwp1-R lateral organs were characterised by ectopic outgrowths on the abaxial surface associated with patches of ectopic adaxial tissue. Leaf margins were particularly affected. The misexpression of adaxial polarity genes in mwp1-R lateral organ primordia is consistent with this phenotype. The most severe phenotypes were seen in the prophylls and silks. It is hypothesised that these organs form via phytomer fusion (Cronquist, 1988; Bossinger et al., 1992; Scanlon and Freeling, 1998). Therefore, the severity of the prophyll and silk phenotypes may be attributable to their each having two sets of margins.

Most of the mwp1-R lateral organs investigated were narrower than wild-type. In some backgrounds mwp1-R lateral organs were also shorter. These observations are consistent with a model in which the adaxial-abaxial boundary promotes growth along both the lateral and proximal-distal axes. A detailed discussion of these results is presented below.

4.4.1 mwp1-R disrupts adaxial-abaxial polarity

mwp1-R husk leaves develop pairs of ectopic outgrowths

mwp1-R husk leaves are characterised by paired flaps of ectopic tissue on the abaxial surface (Figure 4.1, Figure 4.2). These are similar to abaxial margin tissue on their outer surfaces and more similar to adaxial epidermis on their inner surfaces. The orientation of veins also suggests that the tissue between outgrowths has adaxial identity. Veins in mwp1-R outgrowths are oriented with their xylem poles toward the inner surfaces of outgrowths. In some cases veins are partially or completely radialised, with xylem surrounding a central core of phloem, similar to those seen in Arabidopsis phb-1d mutants (McConnell et al., 2001). In situ hybridisation showed that rld1, which is normally expressed on the adaxial side of husk leaf primordia, is misexpressed on the abaxial side of mwp1-R husk leaves, between pairs of outgrowths (Figure 4.3, Figure 4.4). This
result further supports the hypothesis that mwp1-R husk leaves are partially adaxialized.

The mwp1-R phenotype is consistent with the model proposed by Waites and Hudson, in which the juxtaposition of adaxial and abaxial cell types is required for lamina outgrowth (Waites and Hudson, 1995). This model predicts that new boundaries form where a patch of ectopic adaxial tissue is interspersed with normal abaxial tissue, thus initiating a pair of ectopic outgrowths. The margin-like characteristics of mwp1-R husk leaf flaps also support the idea that a boundary between adaxial and abaxial cell types is required for the development of margin characteristics (Sawa et al., 1999). This aspect of the phenotype is common to other polarity mutants, such as Arabidopsis kan1;kan2 mutants, which have leaves that are nearly radial, with marginal cell types found around the entire circumference (Eshed et al., 2004).

Expression of the maize YAB gene, zyb9, was less polarised than rld1 expression in older husk leaves (Figure 4.4). In wild-type husk leaves, expression persists at the margins and in developing vascular bundles but fades in medial regions. zyb9 expression was observed in ectopic outgrowths in medial regions of mwp1-R husk leaves. Expression was strongest in the tip of each outgrowth. This pattern is similar to the misexpression of zyb9 and zyb14 in ectopic outgrowths on lbl1 and Rld1-0 leaf primordia (Juarez et al., 2004a).

Juarez et al. (2004a) observed that zyb expression in older leaf primordia is associated with less determined cell types, such as the margins and central layer of ground tissue. The hypothesis that maize YAB genes promote leaf outgrowth is supported by the observation that ectopic outgrowths on the adaxial side of lbl1 leaf primordia and the abaxial side of Rld1-0 leaf primordia both express zyb9 and zyb14 (Juarez et al., 2004a). Thus, misexpression of zyb9 in mwp1-R husk leaves may be associated with less determined cells and the outgrowth of ectopic laminae, rather than altered polarity per se.

**Single outgrowths develop at the margins of mwp1-R sheaths**
The sheaths of mwp1-R vegetative leaves have phenotypes similar to husk leaves, but are less severely affected. The most consistently observed
phenotype was a single outgrowth immediately adjacent to the sheath margin (Figure 4.15). These outgrowths were confined to the sheath and never extended into the leaf blade. Similar marginal outgrowths occur on husk leaves. The actual margins of affected leaves are blunt, whereas the outgrowths are tapered and look more similar to normal sheath margins. Adaxial-type epidermis extends onto the abaxial side of the leaf to the tip of the outgrowth, so that the adaxial epidermis appears to wrap around the margin (Figure 4.16). Veins in this region are radial and adaxialised. rld1 expression is not confined to the adaxial side of affected leaf primordia, as in wild-type leaves, but is expressed uniformly throughout the leaf margin. Outgrowths develop at the boundary between ectopic rld1 expression and non-expressing cells. These observations are consistent with the idea that the adaxial-abaxial boundary is required for the development of marginal characteristics, as well as for lateral growth (Sawa et al., 1999), as normal margin characteristics develop where there is a distinct adaxial-abaxial boundary. Outgrowths at the margins occur singly, rather than in pairs. The most likely explanation is that only one boundary is created when adaxialised sectors occur at the margins. When adaxial tissue is flanked by abaxial tissue on both sides, two boundaries are created and a pair of outgrowths is initiated.

Outgrowths were frequently observed at the margins of mwp1-R vegetative leaf sheaths that had otherwise normal polarity. This may indicate that adaxial and abaxial domains are specified normally during early development, but are not maintained in mwp1-R sheaths. In maize, differentiation proceeds basipetally and from the midrib to margins (Sharman, 1942). Thus, the sheath margins are the last part of the leaf to differentiate. Based on the mwp1-R phenotype, we predict that Mwp1 may act late in vegetative leaf development to maintain the abaxial domain.

The gram (yab) mutant in Antirrhinum has a leaf margin phenotype that is similar to mwp1-R (Golz et al., 2004). gram leaf margins are blunt and adaxial cell types "wrap around" to the abaxial side. The HD-ZIPIII gene, AmPHB is misexpressed in gram leaves in a similar pattern to rld1 misexpression in mwp1-R mutants. However, outgrowths have not been observed at gram leaf margins. This may be explained if one of the normal functions of GRAM is to
promote lamina outgrowth, as has been proposed for \textit{GRAM} and other \textit{YAB} family members (Siegfried \textit{et al.}, 1999; Eshed \textit{et al.}, 2004; Golz \textit{et al.}, 2004). In \textit{mwp1-R}, \textit{YAB} function is not compromised so could still act to promote ectopic lamina outgrowth.

\textbf{\textit{mwp1-R affects prophyll development in a variety of ways}}

\textit{Normal morphology and development of the prophyll}

Normal prophylls have two outer margins that enclose the ear and two prominent keels that wrap back around the culm (Figure 4.5). The keels are connected by a membranous tissue that lies between the ear and the culm of the mature plant. Epidermal features are similar to regular husk leaves. The adaxial surface is relatively smooth, whereas, the abaxial surface is hairier and the cells are more rounded. In the terminology of Bossinger \textit{et al.} (1992), the prophyll is a type two phytomer - it is a fused organ produced by a newly initiated meristem.

The prophyll is initiated on the side of the lateral meristem adjacent to the main shoot axis and furthest from the subtending leaf (Figure 4.5). The two primordia appear to be initiated simultaneously. At the earliest observable stage the two primordia are connected by a strip of tissue that will form the central membrane (Figure 4.8). These observations support the idea that fusion occurs congenitally (Scanlon and Freeling, 1998). The prophyll grows upward and laterally so that the lateral meristem and subsequent primordia are concealed.

\textit{In situ} hybridisation shows that \textit{rld1} is expressed on the adaxial side of the prophyll (Figure 4.9). Very early in development, a block of \textit{rld1} expression is seen on the side of the prophyll adjacent to the subtending leaf. Cells immediately adjacent to these strongly expressing cells appear to lack \textit{rld1} expression, or to express \textit{rld1} at much lower levels. The boundary between \textit{rld1}-expressing and non-expressing cells extends along the presumptive keel axis (Figure 4.10). This block of expression was not observed in later stage prophylls. We hypothesise that this transient expression pattern may set up a boundary that initiates keel outgrowth. This model is discussed in Section 4.4.8. \textit{rld1} expression persists in the prophyll outer margins and in the central membrane, but often fades from the intervening tissue. It has been observed
previously that rld1 expression persists in vegetative leaf margins (Juarez et al., 2004b). The persistence of rld1 expression in the prophyll central membrane is consistent with the idea that the central membrane represents the fused margins of two leaf primordia (Bossinger et al., 1992; Scanlon and Freeling, 1998).

It was observed that the polarity of individual vascular bundles in wild-type prophyll keels often differed from those in the rest of the prophyll (Figure 4.7). In the outer margins, and in the central membrane, veins were orientated with xylem on the adaxial side and phloem on the abaxial side. Veins in the keel were often rotated relative to veins in flanking regions, and some had two xylem poles. This observation suggests that specification of polarity in the keel region may differ from that of the rest of the prophyll.

*Unfused prophylls are associated with rld1 misexpression early in development*

The most severe manifestation of the mwp1-R phenotype was the reduction of the prophyll to two unfused prongs corresponding to the two midrib regions (Figure 4.6). The central membrane and most of the outer margin regions were deleted. The prongs were usually connected at the very base by a residual strip of membranous tissue. According to the fused phytomer model, the deleted parts correspond to the margin domains of two phytomers. Keel outgrowth was also significantly reduced.

Adaxial-abaxial polarity was distorted in the midrib regions (prongs) of unfused prophylls. Veins were often partially or fully radialised. Analysis of epidermal features indicated that adaxial-type epidermal features continued onto the abaxial side of each prong, so that only the central portion of the abaxial side had normal abaxial epidermal features. This phenotype is reminiscent of the "wrapping around" of epidermal features seen at the sheath margins.

An analysis of prophyll development by SEM indicated that mwp1-R prophylls are initiated normally (Figure 4.8). Early in development the two primordia are connected by a central membrane, similar to wild-type prophylls. By plastochron four there are clear differences between mwp1-R and wild-type prophylls. In the wild-type prophyll the central membrane has elongated and the two tips have
grown laterally. In contrast, the membrane of the mwp1-R prophyll has undergone very little growth and the two tips have elongated, but have not grown laterally. The difference becomes more pronounced later in development. These observations imply that Mwp1 is not required for prophyll initiation, but may act to specify or maintain polarity after emergence. These data are consistent with the idea that the two midrib regions are patterned before the margins.

Measurements of developing prophylls also support the idea that unfused prophylls result from a growth defect that occurs after emergence (Figure 4.11). Early in development, the central membrane regions of mwp1-R and wild-type prophylls are the same height. As the overall height of wild-type prophylls increases the membrane also grows rapidly. In contrast, the central membrane of mwp1-R prophylls grows very slowly relative to overall prophyll height. Given that maturation of maize leaves proceeds basipetally, it seems likely that growth of the basal portion of the primordium contributes most of the height of the prophyll, with the unfused tips differentiating early in development and contributing relatively little to overall height of the mature prophyll. This is supported by the observation that the prophyll tips develop hairs early in development, when the prophyll is less than 3 mm high (see SEMs, Figure 4.11). Wild-type prophylls bifurcate at the very tip (Scanlon and Freeling, 1998). The bifurcated tip is likely to correspond to the unfused portion of the wild-type prophyll primordium.

rld1 is misexpressed early in the development of mwp1-R prophylls that exhibit the unfused phenotype (Figure 4.9). In the central membrane of wild-type prophyll primordia, rld1 expression is confined to the adaxial side. In the corresponding part of early mwp1-R primordia, rld1 is expressed both adaxially and abaxially. This suggests that the adaxial-abaxial boundary is required for lateral and proximal-distal growth of the membrane. Loss of adaxial-abaxial polarity in mwp1-R may result in the loss of growth along the proximal-distal axis. rld1 is also ectopically expressed in a patchy manner in mwp1-R prophyll prongs. This is consistent with the phenotypic analysis which shows distorted polarity. It is likely that loss of distinct adaxial and abaxial domains is
responsible for the lack of proximal-distal growth of the central membrane and lateral growth of the margins.

The mwp1-R prophyll tab phenotype is due to irregular disruption of polarity
A variation of the unfused prophyll phenotype is the development of prophylls that are unfused, but have a tab of tissue extending distally from the central membrane between the two prongs (Figure 4.12). The tabs vary in size and morphology. Some appear to have normal epidermal features, whereas others have outgrowths associated with regions of adaxial identity interspersed with normal abaxial tissue. It seems likely that the phenotypic variation corresponds to irregular disruption of polarity during development. The rld1 expression pattern in prophylls with developing tabs supports this theory. In Figure 4.13, rld1 is misexpressed in the abaxial domain, just below the U-shaped sinuses on either side of the tab. Patchier rld1 expression can be seen in the tab itself and in the region immediately below, but with some retention of a polar expression pattern. This pattern suggests that the development of tabs occurs in regions where some adaxial-abaxial polarity is retained, while growth is retarded in areas where polarity is lost completely. The fact that very long tabs were observed demonstrates that when adaxial and abaxial domains are established, the central membrane region of the prophyll can undergo extensive growth along the proximal-distal axis.

Prophyll fusion defects are due to failure of the central membrane to elongate
Although the most severely affected mwp1-R prophylls are unfused at maturity, my results indicate that the primary defect is a growth defect rather than a fusion defect per se. Firstly, mwp1-R prophylls are initiated normally and the two primordia are connected by a central membrane at the earliest stages. By plastochron 4 the two prongs are physically separated, with connecting membrane only at the very base. Measurement data indicate that the prongs elongate but the central membrane fails to grow (Figure 4.11). Secondly, the tab phenotype demonstrates that the central membrane is capable of significant growth along the proximal-distal axis when adaxial-abaxial polarity is retained. The tab is physically separate from the two prongs. Therefore, it cannot be derived from lateral growth and fusion of the prongs. Figure 4.19 summarises
the *rld1* expression pattern in the central membrane of *mwp1-R* and wild-type prophyll primordia and the corresponding mature prophyll phenotypes.

**Figure 4.19.** Model for establishment of polarity in wild-type and *mwp1-R* prophyll primordia and resulting prophyll phenotypes. (A) In the wild-type prophyll primordium, expression of adaxial factors, such as *rld1*, is confined to the adaxial domain. The adaxial-abaxial axis is specified correctly and the central membrane elongates along with the midrib regions, resulting in a normal fused prophyll. (B) In *mwp1-R* prophyll primordia where adaxial factors are mis-expressed throughout the central membrane, no adaxial-abaxial boundary is created. The midribs grow upward, but the central membrane does not elongate. The result is an unfused prophyll comprising two prongs connected by a strip of membranous tissue at the very base. (C) When expression of adaxial factors is patchy, growth of the central membrane occurs only where an adaxial-abaxial boundary is established, but not in regions where adaxial-abaxial polarity is not retained. The mature prophyll has a tab of membranous tissue flanked by sinuses where the membrane has failed to grow. LM = lateral meristem, P = prophyll primordium.
The mwp1-R subdivided keel phenotype is associated with rld1 misexpression later in development

*mwp1*-R prophylls that develop as fused organs often exhibit severe defects in the keel region (Figure 4.14). This phenotype was most common in the W23 inbred background. In these prophylls, each keel was transformed into a complex series of outgrowths, often with multiple orders of bifurcation. Veins within the keel region were radialized, whereas those in flanking regions had normal polarity. Less pronounced outgrowths were also seen on other parts of the prophyll, similar to those seen on husk leaves. This phenotype suggests that the keel region may be particularly sensitive to disruptions to normal polarity.

*rld1* transcript was detected in the prophyll keels in *mwp1*-R families that express the subdivided keel phenotype, whereas, *rld1* expression was never seen in wild-type keels at equivalent stages (Figure 4.14). *rld1* misexpression occurred much later than in families with unfused prophylls, and tended to be in isolated patches. *rld1* expression was frequently associated with regions where the keel was beginning to divide, suggesting that outgrowths and bifurcations occur at points where polarity is disrupted. The finding that *rld1* misexpression occurs relatively late in development, and that prophylls with subdivided keels are fused suggests that polarity is established correctly, allowing early development to proceed normally. However, polarity is not maintained at later stages and causes aberrant development as the keel is being elaborated. It is plausible that other factor(s) in this background repress *rld1* early in development, but do not act at later stages.

Expression of the maize YAB gene, *zyb9*, was investigated in prophyll primordia exhibiting the subdivided keel phenotype. The results indicate that *zyb9* is misexpressed in *mwp1*-R prophyll keels in a pattern that mirrors *rld1* misexpression. *zyb9* expression was detected in regions where the keel was bifurcated. The YAB genes have been implicated in lamina outgrowth in both dicots and monocots (Siegfried *et al*., 1999; Eshed *et al*., 2004; Golz *et al*., 2004; Juarez *et al*., 2004a). It is likely that misexpression of *zyb9*, and probably other YAB genes, mediates the outgrowth of ectopic laminae in the keel region of *mwp1*-R prophylls.
**mwp1-R silks are most severely affected in marginal domains**

Like the prophyll, the silks are thought to derive from the congenital fusion of two primordia. However, the mode of fusion is different. It is proposed that the two primordia are folded at the midrib and fuse along two sets of margins (Figure 1.6 D). Thus, the two veins correspond to the midribs, and the indented regions correspond to the fused margins (Cronquist, 1988; Scanlon and Freeling, 1998). This model is supported by the finding that in ns mutants, which delete leaf margins, the veins are closer together whereas the distance between the veins and the outer edge of the silk is unchanged (Scanlon and Freeling, 1998).

It was observed that *mwp1-R* silks had the most extensive proliferation of ectopic tissue in the indented regions, the regions corresponding to the fused margins (Figure 4.17). This phenotype is consistent with the folded phytomer model, as the margins are the most severely affected part of other *mwp1-R* organs. Less pronounced outgrowths were seen along the outer edges of silks in the regions corresponding to the midrib domains. The base of the silk, which corresponds to the lower leaf zone, was more severely affected than distal parts (Scanlon and Freeling, 1998).

*mwp1-R* silks often had a kinked or twisted appearance, particularly at the base. Differential growth of opposite sides of the silk is likely to be responsible for this twisting. Epidermal cells were frequently elongated perpendicular to the surface of the silk, suggesting that the plane of cell expansion is disrupted by *mwp1-R*.

### 4.4.2 *mwp1-R* affects lateral and proximal-distal growth

**mwp1-R lateral organs are narrow**

Measurements of *mwp1-R* and wild-type lateral organs showed that *mwp1-R* prophylls, husk leaves, paleae and glumes are significantly narrower than wild-type organs in the W23 background. However, organ length was not affected in this background. In the A188 background, *mwp1-R* prophylls, husk leaves and glumes were significantly narrower and shorter than wild-type organs. The width and length of *mwp1-R* paleae were not significantly different from wild-type
paleae in this background. Vegetative leaf size was not affected by mwp1-R in either the W23 or A188 backgrounds. This may reflect the fact that mwp1-R has only a mild phenotype in the sheaths of vegetative leaves and has no obvious blade phenotype.

Maize ns mutants have narrow leaves that lack normal margin characteristics due to the deletion of a marginal domain (Scanlon et al., 1996; Scanlon and Freeling, 1997; Scanlon, 2000). mwp1-R lateral organs appear to be initiated normally and generally have normal margin characteristics, with the exception of sheaths exhibiting marginal outgrowths. These observations suggest that the narrowness of mwp1-R lateral organs is more likely due to reduced lateral growth than to the deletion of a specific domain.

Narrow lateral organ phenotypes are common in other mutants that affect adaxial-abaxial polarity, indicating that correct specification of the adaxial-abaxial axis is required for lateral growth (Waites and Hudson, 1995; Eshed et al., 2001; Kerstetter et al., 2001; Golz et al., 2004). YAB genes have been implicated in lateral growth and one theory is that the juxtaposition of YAB expressing and non-expressing cells is required to promote growth (Eshed et al., 2004). My results show that rld1 and zyb9 are misexpressed in mwp1-R lateral organs. Thus, the lack of differential YAB expression may contribute to reduced lateral growth of mwp1-R leaves. In normal leaves, there is coordinated cell expansion and division along the lateral axis. In mwp1-R mutants there are multiple ectopic boundaries. Therefore, in these regions cell expansion and division is reoriented along the new axes, at right angles to the plane of the leaf.

The data indicate that mwp1-R affects proximal-distal growth as well as lateral growth. mwp1-R lateral organs in the A188 background were significantly shorter, as well as narrower, than wild-type lateral organs. Growth in both dimensions was similarly affected in this background. In unfused phylls, growth of the central membrane along the proximal-distal axis is severely affected. Reductions in lateral organ length have been observed in other mutants that affect organ polarity (Waites and Hudson, 1995; Eshed et al., 1999; Eshed et al., 2004). It was observed that epidermal cells associated with
ectopic outgrowths at \textit{mwp1-R} sheath margins were shorter than wild-type adaxial sheath margin cells (Figure 4.16). This observation is consistent with the theory that \textit{mwp1-R} disrupts cell elongation along the proximal-distal axis.

It is not surprising that disruptions to the adaxial-abaxial boundary affect proximal-distal growth as well as lateral growth. Although this boundary is frequently depicted as a line, it may be more accurately visualised as a plane of cells between adaxially-specified and abaxially-specified cells (Figure 4.20). Waites and Hudson (1995) describe the boundary as a plate of cells that changes its division pattern to lateral proliferation, thereby forming the lamina. Cells at the boundary could direct cell expansion and division along both the lateral and proximal-distal axes. An analogous mechanism exists in \textit{Drosophila}, where loss of the dorsally expressed gene \textit{apterous} leads to loss of growth in both the proximal-distal and lateral directions (Butterworth and King, 1965).

![Figure 4.20. The adaxial-abaxial boundary promotes both lateral and proximal-distal growth.](image)

\textbf{Fused organs are strongly affected by \textit{mwp1-R}}

\textit{mwp1-R} prophylls exhibit more severe growth defects than husk leaves. In the most extreme cases, the membrane between the two midribs fails to elongate and the prophyll develops as an unfused, two-pronged structure. In the case of
fused prophylls, *mwp1-R* generally had a greater effect on the width of prophylls than on husk leaves in the same background. One possible reason is that leaf margins are particularly affected by *mwp1-R*. As a fused organ, the prophyll has four margin domains, whereas husk leaves have only two. In both the A188 and W23 backgrounds, the distance between the keels was reduced to a similar degree as overall width. This implies a reduction in growth of both the inner and outer margins. An alternative explanation is that organs that are produced earlier are more sensitive to *mwp1-R* than later organs. This would be consistent with the general trend for later husk leaves to be less affected than earlier ones. These two explanations are not mutually exclusive.

Like the prophyll, the palea is a bikeeled organ. It was predicted that, if the palea is a fused organ with four margin domains, then palea width would be more severely affected by *mwp1-R* than glume width. However, *mwp1-R* paleae did not have an obvious phenotype. The size of *mwp1-R* paleae was not significantly different from wild-type paleae in the A188 background. *mwp1-R* paleae were narrower than wild-type paleae in the W23 background. However, they were less affected than glumes in the same background. Scanlon and Freeling (1998) found that the distance between the paleae keels was not significantly reduced in *ns* mutants, although the outer margins were reduced. Thus, their study provided no evidence to support the model that the paleae are fused organs. My results are consistent with this finding.

The silks (stigmas) of *mwp1-R* plants also exhibit altered growth. Measurement data indicate that the veins are closer together in *mwp1-R* silks than in wild-type silks. When interpreted in terms of the folded phytomer model, this phenotype corresponds to a reduction in lateral growth. According to this model, the veins correspond to the two midribs and the indented regions represent the margins of two fused primordia (Cronquist, 1988; Scanlon and Freeling, 1998). Scanlon and Freeling (1998) found that the distance between the veins is reduced in *ns* mutants, whereas, the distance from the veins to the outer edge was unaltered. They also found that *ns* silks lack the surface indentation seen in wild-type silks. The phenotype was interpreted as deletion of the margins. The surface indents were still present in *mwp1-R* silks, although some silks had pronounced
outgrowths in this area. These results point to a reduction in lateral growth in *mwp1-R* silks, rather than deletion of an entire domain.

The distance from the veins to the outer edge of the silk was found to be greater in *mwp1-R* than in wild-type. This could be due to outgrowths in this region, and to the expansion of cells perpendicular to the surface of the silk. This may be due to the loss of adaxial-abaxial polarity and the formation of ectopic boundaries, as is observed in other *mwp1-R* lateral organs.

A number of dicot mutants that affect leaf polarity and growth also have lateral organ fusion defects. In *Antirrhinum*, *gram* mutants often have unfused corolla tubes (Golz *et al.*, 2004). The *gram* phenotype is somewhat similar to *mwp1-R*. *gram* leaves exhibit adaxial-abaxial polarity defects, particularly at the margins, and lateral organs are narrower in *gram* mutants than in wild-type. Thus, the fusion defect may be attributable to reduced lateral growth or to the loss of margin characteristics.

Defects in carpel fusion are seen in *Arabidopsis* mutants of *crabs claw* (*crc*), a YAB gene, and *spatula* (*spt*), a basic-helix-loop-helix transcription factor (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Heisler *et al.*, 2001). *crc* carpels are unfused in the upper third, and this is attributed to the upper part of *crc* carpels being narrower than the lower regions (Alvarez and Smyth, 1999). *SPT* is expressed in the margins of developing carpels and in the septum, a structure derived from the postgenital fusion of outgrowths from the carpel margins (Heisler *et al.*, 2001). These tissues are reduced or lost in *spt-2* mutants, suggesting that *SPT* promotes growth of carpel margin tissue. It is suggested that *SPT* may promote congenital fusion of the carpels directly or, alternatively, the failure to fuse may be a consequence of disrupted lateral growth (Alvarez and Smyth, 1999).

4.4.3 *Mwp1* interacts with networks that establish leaf polarity

Given that *mwp1-R* is a recessive mutation, the adaxialisation of *mwp1-R* lateral organs suggests that *Mwp1* normally acts to promote abaxial identity or to repress adaxial identity. The disruption of vascular polarity in *mwp1-R* lateral
organs suggests a role for Mwp1 in vascular patterning. rld1 is misexpressed in affected organs, indicating that Mwp1 may act to repress rld1 during normal development. In Arabidopsis, an antagonistic relationship between HD-ZIPIII and KAN family members functions in the establishment of polarity in lateral organs and vascular tissue (Eshed et al., 2001; Kerstetter et al., 2001; Emery et al., 2003; Eshed et al., 2004).

Henderson et al. (2006) found that the maize KAN gene, Zm*KAN2, is expressed in a complementary pattern to rld1. Their finding is consistent with, although does not prove, an antagonistic interaction between maize KANs and HD-ZIPIII genes. Analysis of KAN expression in a Rld1 mutant background would help clarify whether such an antagonistic relationship exists.

miRNA165/166 has been shown to regulate rld1 expression in maize (Juarez et al., 2004b). Indeed, miRNA-directed cleavage of HD-ZIPIII mRNAs is conserved throughout the land plants (Floyd and Bowman, 2004). In addition, there is evidence that rld1 expression is controlled in part at the transcriptional level (Juarez et al., 2004b). KAN genes, such as Mwp1, may act at this level.

There are several scenarios as to how HD-ZIPIII and KAN genes might interact to specify adaxial and abaxial domains. Firstly, there could be an antagonistic relationship, with HD-ZIPIII genes specifying adaxial fate, and KANs specifying abaxial fate. In mwp1-R mutants, a loss of KAN function allows HD-ZIPIII misexpression on the abaxial side of leaf primordia. Reduced levels of KAN coupled with ectopic HD-ZIPIII expression would then result in cells in these regions adopting adaxial identity. Alternatively, adaxial and abaxial domains may be determined by regions of HD-ZIPIII expression and non-expression respectively. In this scenario, the main role of KAN genes would be to repress HD-ZIPIII expression rather than to specify abaxial fate directly. A similar mechanism has been proposed to pattern Arabidopsis vasculature (Emery et al., 2003). Finally, it may be the relative levels of HD-ZIPIII and other factors that determine cell identity. This model is supported by the finding that misexpression of rld1 is sufficient to switch adaxial and abaxial cell identities (Nelson et al., 2002).
Maize YAB (zyb) genes are expressed in lateral organs in a similar pattern to rld1 (Juarez et al., 2004a), although my data indicate that zyb9 expression in older husk leaves is less polarised than rld1. zyb expression is increased in Rld1 mutants, suggesting that zyb genes are downstream of rld1 (Juarez et al., 2004a). However, Rld1 misexpression is not sufficient to induce zyb expression on the abaxial side of young Rld1 primordia, suggesting that other factors, in addition to HD-ZIPIII genes, control zyb expression (Juarez et al., 2004a).

My results show that zyb9 is misexpressed in mwp1-R lateral organs, in a pattern that mirrors rld1 misexpression. This pattern could indicate that YAB genes are downstream of HD-ZIPIII genes in maize, as suggested by Juarez et al. (2004b). Alternatively, Mwp1 may repress zyb expression directly. Thus, Mwp1 and other KAN genes may constitute the additional factors controlling zyb expression. It is possible that zyb genes are positively regulated by HD-ZIPIII genes and negatively regulated by KAN genes. The current results do not allow me to distinguish between these possibilities.

rld1 is misexpressed in both mwp1-R and Rld1 mutants, and both mutants show adaxialisation of abaxial tissues (Nelson et al., 2002; Juarez et al., 2004b). However, there are differences between the mutant phenotypes. rld1 causes adaxialisation of sectors of the blade, whereas, the mwp1-R phenotype is generally confined to sheath tissue. In mwp1-R, vegetative leaves are less severely affected than husk leaves, whereas, no husk leaf phenotype has been described for Rld1 mutants. Rld1 leaves sometimes exhibit a switching of adaxial and abaxial epidermal identities (Nelson et al., 2002). This was not observed in mwp1-R mutants.

There are a number of factors that may account for these phenotypic differences. Firstly, the redundant activity of additional KAN family members may mean that rld1 is only misexpressed in specific tissues in mwp1-R mutants. Secondly, the temporal and spatial patterns and the level of rld1 misexpression are likely to differ in the two mutants. In mwp1-R leaf primordia, rld1 is misexpressed in patches, whereas, in Rld1-O mutants, rld1 is misexpressed in a more uniform manner (Juarez et al., 2004b). In Rld1 mutants, rld1 misexpression is due to loss of regulation by miRNAs, whereas, in mwp1-R
mutants *rld1* misexpression is due to the loss of one *KAN* function. Thirdly, in *Rld1* mutants only a single *HD-ZIPIII* gene is misexpressed, whilst it is possible that multiple *HD-ZIPIII* genes are misexpressed in *mwp1-R* mutants.

*mwp1-R* mutants exhibit a more severe disruption of vascular polarity than has been reported for *Rld1* mutants. Veins at the junctions of *mwp1-R* husk leaf outgrowths and sheath margins may be partially or completely radialised, whereas *Rld1* major veins are unaffected and minor veins may be lost or mis-oriented (Nelson et al., 2002; Juarez et al., 2004a). In wild-type leaf primordia, *rld1* is expressed in immature vascular strands and becomes localised to pro-xylem cells (Juarez et al., 2004b). Expression in vascular bundles is not altered by loss of *lbi1* function (Juarez et al., 2004a). Given that *Lbi1* operates in the miRNA pathway (per. comm. Marja Timmermans), this finding suggests that *rld1* is regulated independently of miR165/166 in vascular bundles. Thus, *rld1* expression may be relatively normal in *Rld1* vascular bundles, but misexpressed in *mwp1-R* vascular bundles. It is likely also that *HD-ZIPIII* genes other than *rld1* are misexpressed in *mwp1-R* leaves, and these may contribute to vascular patterning. For example, a maize *PHB* homologue is expressed specifically in pro-xylem cells during normal development (Juarez et al., 2004b).

### 4.4.4 Expression of the mwp1-R phenotype varies in different inbred backgrounds

Maize is genetically very diverse. Mutant phenotypes may vary in different backgrounds due to the variability of modifying genetic factors and differences in developmental rate (Bertrand-Garcia and Freeling, 1991; Tenaillon et al., 2001; Fu and Donner, 2002; Song and Messing, 2003; Brunner et al., 2005). The expression levels of homologous genes have been found to differ in different inbred backgrounds (Song and Messing, 2003; Guo et al., 2004). Thus, the pattern and level of expression of genes in interacting networks is likely to influence mutant phenotypes in different backgrounds. There are also morphological differences between inbred lines. For example, the size of the SAM varies in different inbred lines and is correlated with penetrance of the *kn1-E1* phenotype (Vollbrecht et al., 2000).
The *mwp1-R* phenotype varies depending on the background in which it was expressed. The phenotype is very mild in the B73 background, so was not examined in detail in this background (per. comm., Hector Candela and Sarah Hake). B73 is a late flowering line (Bertrand-Garcia and Freeling, 1991). It is possible that additional factors expressed later in development could compensate for loss of *Mwp1* function in this background.

The prophyll phenotype shows considerable variation depending on background. These differences are correlated with temporal differences in *rld1* misexpression. In its most severe form, the prophyll is unfused. This phenotype was most consistently observed in a non-introgressed background, and occasionally in the W23 background. The unfused prophyll phenotype is associated with *rld1* misexpression early in development. In the W23 background, the most commonly observed prophyll defect was the subdivision of the keel into a series of outgrowths. This subdivided keel phenotype is associated with patchy misexpression of *rld1* later in development.

The *mwp1-R* subdivided keel phenotype was seldom seen in the A188 background. A188 is a fast-maturing line. One possibility is that prophyll primordia in the A188 background reach a later stage of differentiation before polarity is disrupted by loss of *Mwp1* function. Such disruptions may occur too late to affect morphogenesis, but could still affect subsequent growth. Alternatively, there may be subtle differences in the way *HD-ZIPIII* genes are regulated in different inbred backgrounds.

The effects of *mwp1-R* on lateral organ growth also differed in A188 and W23. In general, in the W23 background *mwp1-R* lateral organs were reduced in width, but organ length was not significantly different. In the A188 background, both the width and length of *mwp1-R* lateral organs were reduced. The reduction in width was generally greater in the W23 background. The adaxial-abaxial boundary can be conceived as a plate of cells that directs growth along the lateral and proximal-distal axes (Figure 4.20). It is likely that multiple factors are involved in setting up this boundary and in directing growth along the axes. There may be subtle differences in these networks that account for the different growth defects in the W23 and A188 backgrounds. The expression of potential
interacting genes would need to be studied in more detail before any conclusions could be drawn about the effects of background-dependent factors on the mwp1-R phenotype.

4.4.5 mwp1-R specifically affects sheath tissue

mwp1-R affects a range of lateral organs. However, the phenotype is generally confined to sheath tissue and husk leaf phenotypes are more severe than vegetative leaf phenotypes. Given that there are at least 11 KAN genes in maize, it is likely that there is a high level of redundancy and subfunctionalisation in this gene family (per. comm., Hector Candela and Sarah Hake). Thus, Mwp1 may act primarily in sheath tissue, with other KAN family members fulfilling a similar role in blade tissue. Likewise, Mwp1 may be more important in husk leaf development, with additional KANs acting in a partially redundant manner during vegetative leaf development.

In Arabidopsis there are four KAN genes, and they act in a partially redundant manner. Single mutants have very mild phenotypes compared to double and triple kan mutants (Kerstetter et al., 2001; Eshed et al., 2004). KAN4 expression is limited to ovules and is required for development of the inner integument, whilst KAN1 and KAN2 act redundantly to provide a homologous function in the outer integument (McAbee et al., 2006). KAN genes in maize may have similarly specialised functions, indeed there is likely to be a higher level of subfunctionalisation due to the larger maize KAN family.

4.4.6 mwp1-R interacts with proximal-distal patterning mutants

mwp1-R phenotypes are generally confined to the sheath or basal parts of lateral organs. However, blade tissue adjacent to ectopic sheath-like tissue in mwp1-R;Wab1-R double mutants also exhibits disrupted adaxial-abaxial polarity (Figure 4.18). This was not observed in Wab1-R single mutants. Blade tissue adjacent to ectopic sheath tissue exhibited disrupted adaxial-abaxial polarity, with bulliform cells and macrohairs on the abaxial surface and misoriented or radialised vascular bundles. Similar phenotypes are seen in mwp1-R;Rs1-R and mwp1-R;Kn1-R double mutants, indicating that this interaction isn't specific to Wab1-R. One explanation is that Mwp1 is specifically required for patterning of
sheath tissue or basal tissue types. There may be blade-specific KAN genes that act in normal blade tissue, but are not expressed in ectopic sheath tissue. It is possible that, once incorrect adaxial-abaxial patterning is established in ectopic sheath tissue, this positional information is transmitted to adjacent blade tissue.

4.4.7 Comparison of grass and dicot modes of growth

Differences in the phenotypes of dicot and grass leaf polarity mutants may reflect differences in the way that leaves are initiated and elaborated. In dicots, a number of mutants that affect adaxial-abaxial polarity have leaves that are radial or almost radial. The leaf primordia of dicots such as tobacco emerge as peg-like structures. The lamina is initiated subsequently from cells on the flanks of the primordium (Poethig and Sussex, 1985a). In maize, the leaf lateral axis is established within the SAM and the primordium has a flattened lamina at the time of emergence. Only mutations that affect founder cell recruitment, such as lb1, result in radial leaves (Timmermans et al., 1998).

Elaboration and differentiation of maize leaves proceeds basipetally and from the midrib to margins. Thus, the sheath margins are the last part of the leaf to differentiate (Sharman, 1942; Sylvester et al., 1996; Scanlon, 2003). mwp1-R lateral organs are generally most affected in the sheath margin domains. One possible explanation is that Mwp1 is required to maintain abaxial identity at later stages of development. Maize leaves grow for a more prolonged period than many dicot leaves. In maize, growth persists at the margins and contributes substantially to the width of the leaf base. This is evidenced by wide marginal clonal sectors (Poethig, 1984; Poethig and Szymkowiak, 1995). In dicots, the margin plays only a minor role in growth of the lamina. Cells at the margins begin differentiation early in leaf development and marginal clonal sectors are relatively narrow (Poethig and Sussex, 1985a; Poethig and Sussex, 1985b). These differences in growth patterns may explain why mwp1-R phenotypes are most pronounced in margin domains, whereas, in dicots, the leaf margins do not show particularly strong kan phenotypes.
Mutations that specifically affect either the upper or lower leaf zone may also manifest differently, as the maize leaf is comprised almost entirely of lower leaf zone, whereas the dicot leaf is mainly upper leaf zone (Figure 1.7) (Troll, 1955; Kaplan, 1973; Nardmann et al., 2004). mwp1-R defects are generally specific to sheath tissue. The sheath corresponds to the basal portion of the lower leaf zone. Therefore, equivalent defects in dicot leaves may be difficult to detect, as the lower leaf zone forms only a minor part of the mature leaf.

Outgrowths on mwp1-R leaves tend to occur as long flaps that run parallel to lateral veins, whereas, outgrowths on the leaves of Arabidopsis kan mutants are radial (Eshed et al., 2004). A linear arrangement of tissues is characteristic of grass leaves (Sylvester et al., 1996). Lateral veins form parallel to the midrib and clonal sectors are generally confined along vascular boundaries (Cerioli et al., 1994). In dicots, veins are initiated at an oblique angle to the midrib and clonal sectors tend to be isodiametric (Poethig and Sussex, 1985a; Poethig and Sussex, 1985b). In dicots, cells expand isotropically, whereas, in grass leaves, cell expansion is polarised longitudinally (Poethig, 1984). Differences between mwp1-R and Arabidopsis kan phenotypes may reflect differences in the structure and development of Arabidopsis and maize leaves.

4.4.8 Prophyll morphogenesis

**Do existing meristems and leaf primordia influence the development of organs on newly ramified meristems?**

The prophyll is the first organ initiated by the newly formed lateral meristem and it is likely that this affects its development and morphology. Unlike subsequent husk leaves, the prophyll is not subject to the influence of existing primordia. The prophyll arises on the adaxial side of the lateral meristem. Thus, it may be subject to the influence of two meristems – the lateral meristem from which it was initiated and the SAM of the main axis. It is unclear how much influence the SAM would exert on the developing prophyll as, by the time the prophyll is initiated by the lateral meristem, the SAM has initiated several additional vegetative leaves.
It is possible that prophyll development is influenced by the proximity of newly initiated vegetative leaves rather than, or in addition to, the SAM. During the early stages of prophyll development, the main shoot axis is compressed and the lateral meristem exists in close proximity to distal primordia of the main axis and to the subtending leaf. Bossinger et al. (1992) suggest that the morphology and topology of the first organs initiated by newly ramified meristems (type 2 phytomers) may be influenced by existing leaf primordia. During initiation of the lateral meristem and early prophyll development, the subtending vegetative leaf and leaves at distal nodes express \textit{rld1} and \textit{zyb9}. In addition \textit{Zm*KAN2} is expressed in leaf primordia and in the disk of insertion (Henderson et al., 2006). These, or other factors expressed by leaves adjacent to the lateral meristem may influence prophyll development.

The shape of the lateral meristem may also influence prophyll development. Meristem shape and size are known to influence phyllotaxy, for example in the maize \textit{abphy1} mutant (Jackson and Hake, 1999; Giulini et al., 2004). The newly initiated lateral meristem is sandwiched between the main axis and the subtending leaf and is elliptical in transverse section. As primordia are initiated, the meristem assumes a more rounded shape. It is possible that the elongated shape of the meristem causes the initiation of two primordia simultaneously. This theory is consistent with the observation that newly formed floret meristems are somewhat elliptical in shape and initiate paleae, which are also fused organs (Bonnett, 1940; Cheng et al., 1983).

Auxin flux in the SAM has been shown to be a crucial factor in leaf initiation and phyllotaxis. According to auxin flux models, existing primordia act as auxin sinks leading to auxin maxima at regions furthest from existing primordia. New primordia arise in these areas of highest auxin concentration (Reinhardt et al., 2000; Reinhardt et al., 2003). Auxin response factors have been implicated in adaxial-abaxial patterning and lamina outgrowth, suggesting that auxin may also function in patterning of lateral organs (Pekker et al., 2005). It is likely that auxin is involved in the unique topology and morphology of lateral organs produced by newly formed meristems. One possibility is that leaf primordia that flank the new lateral meristem could influence auxin flux in the lateral meristem and thus determine the positioning of prophyll primordia. Auxin may also

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mediate fusion of the two primordia, as auxin application has been shown to cause fusion of leaf primordia and inhibition of auxin transport results in fusion of sheath margins in maize (Snow and Snow, 1937; Scanlon, 2003). It should be emphasised that these suggestions are speculative. An investigation of auxin flux in lateral meristems and the localisation of associated proteins may elucidate this matter.

The keel is sensitive to disrupted polarity
The maize prophyll has two prominent keels that partially encircle the culm. The keels correspond to the midrib regions of two fused leaves. The keel region is particularly sensitive to disrupted polarity caused by mwp1-R. In families where the prophyll is unfused, keel outgrowth is dramatically reduced. In families where mwp1-R prophylls remain fused, a phenotype is often observed where each keel is reduced to a convoluted series of outgrowths. The observation that individual veins in wild-type keels are often oriented differently to veins in surrounding tissue, and the dynamic expression pattern of rld1 in the keel region, suggest that polarity may be established differently in the keel than in adjacent regions.

Model for keel outgrowth
We speculate that the adaxial-abaxial patterning system has been co-opted during evolution to promote outgrowth of the keels in normal prophyll development. A model is presented in which a pulse of adaxial identity on the abaxial side of the prophyll generates a new boundary that initiates keel outgrowth (Figure 4.21). In mwp1-R prophylls, ectopic expression of adaxial factors means that no distinct boundary is created. Thus, keel outgrowth is significantly reduced.

According to this model, adaxial (blue) and abaxial (yellow) domains are established in the two primordia that comprise the prophyll. This boundary between adaxial and abaxial domains generates the lateral axis. Subsequently, adaxial factor(s) are expressed briefly at the outer edge of each primordium on the abaxial side (stippled). These blocks of adaxial identity set up additional boundaries at right angles to the lateral axis, thereby initiating keel outgrowth. The blocks of rld1 expression that are seen at the outer edges of early stage
prophylls may correspond to this localised pulse of adaxial identity. The boundary between *rld1*-expressing and non-expressing cells in early stage prophyll primordia aligns with the axis of the emerging keel. The observation that individual veins in wild-type prophyll keels often have a different orientation to surrounding veins is also consistent with this model.

This model could explain the reduction in keel outgrowth seen in unfused *mwp1-R* prophylls. According to the model, adaxial and abaxial domains are initiated normally in *mwp1-R* prophylls. Thus, *mwp1-R* prophylls appear normal shortly after initiation. Without *Mwp1* function, abaxial identity is not maintained. Consequently, there is no strong boundary to promote keel outgrowth. The lateral boundary is also less distinct and, as a result, lateral growth is much reduced.

![Diagram](image)

Figure 4.21. Model for outgrowth of the prophyll keels. (A) In wild-type prophyll development, adaxial-abaxial polarity is established within the meristem (grey) and maintained after emergence. We speculate that keel outgrowth is promoted by a transient localised pulse of adaxial-promoting factor(s) (stippled) on the abaxial side. This creates a boundary (red) that promotes outgrowth of the keel (red arrows) at right angles to the lateral axis. Lateral growth (black arrows) contributes to the outer margins and central membrane of the prophyll. (B) In *mwp1-R* prophyll primordia, abaxial identity is not maintained. No boundaries are created to promote keel outgrowth, and lateral growth is significantly reduced (grey arrows). LM = lateral meristem.
Analysis of *mwp1*-R prophylls that exhibit the subdivided keel phenotype suggests that the keel region is particularly sensitive to disruptions to abaxial identity. The model for keel outgrowth may explain why the keel is more severely affected than surrounding regions. If the keel axis is established by the transient expression of adaxial identity genes on the abaxial side, then this early exposure to adaxial factors may make cells in the keel region more susceptible to ectopic adaxial factors later in development. The resulting patches of adaxialised cells interspersed with abaxial cells would create multiple ectopic boundaries, generating a complex array of outgrowths.
5. Conclusions and future work

5.1 Conclusions

A fundamental developmental mechanism is the subdivision of fields of cells into smaller compartments and the concomitant initiation of new axes of growth (Diaz-Benjumea and Cohen, 1993; Lawrence and Struhl, 1996). This study focuses on three mutants, Wab1-R, lg1-R and mwp1-R that disrupt axial patterning of maize leaves. Analyses of mutant phenotypes and gene expression patterns, and a series of mosaic analyses have uncovered interactions between factors involved in defining developmental compartments and growth axes during maize leaf development.

Wab1-R is a dominant mutation that disrupts proximal-distal patterning of maize leaves, resulting in ectopic auricle and sheath tissue in the leaf blade and narrow leaves (Hay and Hake, 2004). Both aspects of the phenotype are exacerbated by lg1-R. A mosaic analysis of Wab1-R indicated that Wab1-R generally acts cell-autonomously. Examples of Wab1-R non-autonomy were only observed in a Lg1 background, supporting a role for Lg1 in signal propagation. We suggest that Lg1 can transmit positional information from Wab1-R tissue into wab1- tissue and from wab1- tissue into Wab1-R tissue.

Leaf shape and positioning of the blade-sheath boundary are altered in lg1-R mutants, implying a role for Lg1 in modulating leaf shape. Becraft et al. (1990) found that Lg1 acts cell-autonomously to specify ligule and auricle tissue. My results indicate that Lg1 also conditions Wab1-R ectopic auricle tissue in a cell-autonomous manner. However, the data suggest that Lg1 promotes lateral growth non-autonomously. Thus, Lg1 has both cell-autonomous and non-autonomous functions. These functions may be mediated by separate downstream pathways.

Hay and Hake (2004) proposed that the Wab1-R narrow leaf phenotype is due to the deletion of a lateral domain. However, a clonal analysis of Wab1-R leaves presented in this thesis does not support this hypothesis. Wab1-R leaf primordia
appear narrower than wild-type leaf primordia at plastochron three (Hay and Hake, 2004). A comparison of lateral vein initiation in Wab1-R and wild-type leaf primordia found a difference in lateral vein number one plastochron later than the observed difference in width. This finding is consistent with a space-filling model rather than a pre-pattern model for vascular initiation.

Analysis of the mwp1-R phenotype revealed that adaxial cell-types on the abaxial side of mwp1-R lateral organs are associated with ectopic outgrowths. The phenotype is generally confined to sheath tissue. Genes that are normally expressed adaxially are expressed on the abaxial side of mwp1-R lateral organs. The mwp1-R phenotype is consistent with a model in which the juxtaposition of adaxial and abaxial compartments generates a new axis and promotes lateral growth along this axis (Waites and Hudson, 1995). Given that mwp1-R is a recessive mutation, these results suggest that Mwp1 is required for the establishment or maintenance of abaxial identity in sheath tissue.

The margins of mwp1-R lateral organs exhibit the most severe phenotypes. The sheath margin phenotype is consistent with the idea that correct establishment of adaxial-abaxial polarity is required for the development of normal margin characteristics (Sawa et al., 1999). The fact that mwp1-R specifically affects sheath tissue and the phenotype is most pronounced at the margins suggests that Mwp1 acts relatively late in development, as the sheath margins are the last part of the leaf to differentiate. It is likely that other KAN family members perform similar functions in other parts of the leaf or at different stages of development.

mwp1-R affects most lateral organs. This supports the concept that common genetic programmes are involved in the patterning of all lateral organs, and implies a shared evolutionary origin (Bossinger et al., 1992). Phenotypic differences are likely to reflect differences in lateral organ morphogenesis. For example, vegetative leaves were most consistently affected at the sheath margins, whereas, the silks had the most severe outgrowths in the central indented regions. The silk phenotype is consistent with the fused phytomer model, in which the indents correspond to the fused margins of two highly modified leaves (Cronquist, 1988; Scanlon and Freeling, 1998).
The prophyll exhibits the most severe *mwp1-R* phenotypes and is also considered to be a fused organ (Bossinger *et al.*, 1992; Scanlon and Freeling, 1998). The prophyll has an unusual morphology, with two prominent keels corresponding to the two midrib domains. The keel region is particularly sensitive to disruptions to adaxial-abaxial polarity by *mwp1-R*. We propose that the adaxial-abaxial patterning system has been co-opted to promote outgrowth of the keels during normal prophyll development (Figure 4.21). Evidence to support this model includes the disruption of keel development by *mwp1-R*, the transient boundary of *rld1* expression along the keel axis and the different orientation of individual veins in the keel region compared to other parts of the prophyll.

Analysis of the *Wab1-R* and *mwp1-R* mutant phenotypes provides evidence that the axes of growth are patterned interdependently. Disruption of either the proximal-distal axis in *Wab1-R* or the adaxial-abaxial axis in *mwp1-R* reduces lateral growth along the medial-lateral axis. In some backgrounds, *mwp1-R* also causes a reduction in growth along the proximal-distal axis. Analysis of the *mwp1-R* "tab" phenotype provides further evidence that adaxial-abaxial patterning is required for proximal-distal growth. The central membrane of the prophyll can undergo extensive growth along the proximal-distal axis in regions where adaxial-abaxial polarity is specified correctly, but not in adjacent regions where polarity is mis-specified. This makes sense if the adaxial-abaxial boundary is conceptualised as a plate of cells that co-ordinates cell division and expansion along both the medial-lateral and proximal-distal axes (Figure 4.20) (Waites and Hudson, 1995).

Correct specification of developmental compartments is crucial for normal leaf development. The axes of growth establish the planes of cell division and expansion, which in turn direct lateral organ morphogenesis. When these axes are disrupted, the final shape of the leaf is altered. Positional information is also required for the appropriate differentiation of specific tissues. As well as the delimitation of compartments and the specification of cell-types, there is a requirement for inter-compartmental signalling to ensure co-ordinated growth of the leaf. Thus, both cell-autonomous and non-autonomous pathways are
necessary for leaf development. The results of this study place \textit{Mwp1}, \textit{wab1} and \textit{Lg1} in a network of genes that regulate leaf polarity and axial patterning.

### 5.2 Future work

The identity of \textit{wab1} is not yet known. Cloning of \textit{wab1} will facilitate investigations of \textit{wab1} function. It will enable \textit{in situ} hybridisation to determine the \textit{wab1} expression pattern during normal leaf development and in \textit{Wab1} mutants, as well as interactions with other genes. Identifying the molecular lesion(s) in \textit{Wab1} alleles may provide information about the regulation of \textit{wab1}, or about the function of WAB1 protein. Obtaining a \textit{wab1} loss-of-function mutant and analysis of the mutant phenotype will be crucial to determining the normal function of \textit{wab1}.

\textit{Mwp1} has recently been cloned and is a member of the \textit{KAN} gene family. Work is currently underway to characterise the \textit{Mwp1} expression pattern. In light of the severe \textit{mwp1-R} phenotypes in prophylls and silks, it will be particularly interesting to characterise the \textit{Mwp1} expression pattern in these organs. In \textit{Arabidopsis}, the \textit{KAN} and \textit{HD-ZIPIII} genes have an antagonistic relationship (Eshed \textit{et al.}, 2001; Kerstetter \textit{et al.}, 2001; Emery \textit{et al.}, 2003). We have shown that \textit{rld1} is ectopically expressed in \textit{mwp1-R} mutant leaves. It is likely that other \textit{HD-ZIPIII} genes are also misexpressed in \textit{mwp1-R}. This could be investigated by analysing the expression patterns of other maize \textit{HD-ZIPIII} genes in wild-type and \textit{mwp1-R} lateral organs. Analysis of the \textit{Mwp1} expression pattern in \textit{Rld1} mutants will help determine if an antagonistic relationship between the \textit{KAN} and \textit{HD-ZIPIII} genes exists in maize.

There are at least 11 \textit{KAN} genes in maize. Therefore, there is likely to be a high level of redundancy. Obtaining multiple \textit{kan} loss-of-function mutants may reveal phenotypes that would be obscured by redundancy in single mutants. In particular, blade tissue is generally not affected by \textit{mwp1-R}. It will be interesting to see whether blade phenotypes are observed in other \textit{kan} mutants.

Auxin has been implicated in lateral organ initiation and in leaf polarity (Reinhardt \textit{et al.}, 2000; Reinhardt \textit{et al.}, 2003; Pekker \textit{et al.}, 2005). Auxin flux is
mediated by PIN proteins (Galweiler et al., 1998). Therefore, it may be informative to investigate the pattern of PIN localisation in ectopic outgrowths during mwp1-R leaf development and during initiation of the prophyll keel. The prophyll is characteristic of a type two phytomer in that two primordia are initiated simultaneously by a newly ramified meristem (Bossinger et al., 1992). Factors that may influence this unique topology include the elliptical shape of the meristem, and the influence of flanking meristems and leaf primordia. Given that auxin levels determine the positioning of new primordia, it will be interesting to study PIN localisation during prophyll initiation and compare this with initiation of subsequent husk leaves.

We propose that the adaxial-abaxial patterning system has been co-opted to promote outgrowth of the keels during normal prophyll development. This could be investigated further by determining the expression patterns of other polarity genes during normal prophyll development, and by characterising any prophyll phenotypes conditioned by other mutants that disrupt leaf polarity. It may also be informative to analyse the Mwp1 gene to identify motifs that regulate the Mwp1 expression pattern in the prophyll.

It is possible that the adaxial-abaxial patterning network has been co-opted to pattern unusual leaf forms in other species. This could be addressed by investigating the function of homologues of known polarity genes in species with diverse lateral organ morphologies.
References


