GRAMINIFOLIA promotes growth and polarity of Antirrhinum leaves

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Summary

The leaves of higher plants develop distinct cell types along their adaxial-abaxial (dorsal-ventral) axes. Interaction between leaf primordium cells with adaxial and abaxial identities is necessary for lateral growth of the developing leaf blade. We show that the growth and asymmetry of leaves in Antirrhinum majus involves the related YABBY transcription factors GRAMINIFOLIA (GRAM) and PROLONGATA (PROL). GRAM is expressed in abaxial margins of organ primordia where it promotes lateral growth and abaxial cell fate. GRAM, however, is not needed for abaxial fate in the absence of adaxial cell specification, suggesting that it promotes abaxial fate by excluding adaxial identity. Although GRAM expression is abaxially restricted, it functions redundantly with its abaxially expressed parologue, PROL, and with the ubiquitously expressed PHANTASTICA gene to promote adaxial identity via intercellular signalling. This non cell-autonomous behaviour is consistent with the ability of GRAM in only the abaxial most cell layer to direct normal development of more adaxial cells. The contrasting roles of GRAM in promoting and inhibiting adaxial identity might serve to reinforce and maintain the distinction between adaxial and abaxial domains in the growing leaf primordium.

Key words: Antirrhinum majus, GRAMINIFOLIA, PROLONGATA, YABBY, Leaf asymmetry

Introduction

As a leaf primordium emerges from the periphery of the shoot apical meristem (SAM) it flattens perpendicular to its adaxial-abaxial axis and subsequently develops layers of functionally specialised cell types asymmetrically along this axis. The growth that flattens the organ occurs around the ad-abaxial boundary, is abolished in mutants that have lost either identity and occurs ectopically at novel boundaries (Eshed et al., 2001; Kerstetter et al., 2001; Kim et al., 2003; McConnell and Reinhart et al., 2002; Rhoades et al., 2002). Consistent with this model is the finding that these miRNAs accumulate in the abaxial domain of Arabidopsis and maize lateral organs (Juarez et al., 2004; Kidner and Martienssen, 2004). Because transcripts from the gain-of-function alleles no longer match the miRNA perfectly and are resistant to degradation (Tang et al., 2003), they might persist in the abaxial domain to specify ectopic adaxial fate. This second model does not exclude the possibility that the HD-ZIP proteins are also activated by a ligand. However, these models make different assumptions about how organ asymmetry is first specified. Adaxial HD-ZIP activation by a ligand from the centre of the SAM could constitute the first step in organ polarisation, whereas inactivation by the abaxially localised miRNA implies that the organ is already polarised or that the miRNA is itself the polarising signal.

KANADI (KAN) genes, which are both necessary and sufficient for abaxial fate in Arabidopsis leaves (Eshed et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001), are needed to limit HD-ZIP gene expression to an adaxial domain (Eshed et al., 2001). Because loss of PHB, PHV and REV activity has a

(Reinhart et al., 2001). This suggests that a juxtaposition of cells with adaxial and abaxial identities in a developing leaf is required for lateral growth, presumably through cell-cell signalling. This same mechanism is likely to control ad-abaxial asymmetry and growth in other lateral organs, such as bracts and petals.

In Arabidopsis, the related HD-ZIP genes PHABULOSA (PHB), PHAVOLUTA (PHV) and REVOLUTA (REV) specify adaxial leaf identity. The activity of these genes normally becomes restricted to the adaxial domain of newly initiated leaf primordia (McConnell et al., 2001; Otsuga et al., 2001). The characterisation of gain-of-function PHB and PHV alleles, which result in their ectopic abaxial expression and adaxial fate, have suggested two mechanisms that might normally limit PHB and PHV activity to the adaxial domain of developing organs. One proposes that the HD-ZIP proteins are activated by binding a ligand adaxially, promoting their own expression so that gain-of-function mutations, which affect the potential ligand binding site, render the proteins constitutively active (McConnell et al., 2001). The hypothetical ligand may come from the centre of the SAM, because leaf initials surgically isolated from the SAM fail to form adaxial cell types (Sussex, 1955). The second explanation is that a short microRNA (miRNA) complementary to wild-type RNA from the HD-ZIP loci causes degradation of PHB and PHV, and possibly REV, transcripts in the abaxial leaf domain (Emery et al., 2003; Reinhart et al., 2002; Rhoades et al., 2002). Consistent with this model is the inactivation by the abaxially localised miRNA implies that the organ is already polarised or that the miRNA is itself the polarising signal.

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Materials and methods

Plant material

The amyb\textsuperscript{constans} (gram-1) and amyb\textsuperscript{mutabilis} (gram-2) mutants (Baur, 1918) and the MAM265 line (Stubbe, 1966) came from the IPK, Gatersleben, Germany. The gram-3 mutant arose in the transposon-active line JI.75 (Carpenter and Coen, 1990), and was a gift from Rosemary Carpenter and Enrico Coen (John Innes Centre, UK). It was shown to carry a single recessive mutation, gram-3, that failed to complement gram-1 or gram-2.

Periclinical chimeras for an olive (oli) mutation, which prevents accumulation of chlorophyll at higher light intensities (Hudson et al., 1993), were generated from the unstable oli-605 allele in gram-1 and GRAM\textsuperscript{+} backgrounds. Plants were grown at 15°C for 10 days to induce excision of the Tam\textsuperscript{3} transposon from oli-605, then maintained at 25°C, to inhibit further transposition, in a light intensity of ~200 μmol/m²-second, to distinguish OLI\textsuperscript{+} revertant and oli mutant tissue. Chlorophyll was identified in hand-cut sections by epifluorescence at 365 nm excitation. Periclinical chimeras were maintained and propagated vegetatively from cuttings.

The branch of either phan-249 × gram-1 (in the Sippe 50 genetic background), or phan-607 × gram-3 (in the JI.75 background). About 6% of F\textsubscript{2} progeny showed an enhanced mutant phenotype that included lack of an embryonic apical meristem. These plants were confirmed as gram phan double mutants by Southern hybridisation, gram prol and phan prol double mutants were obtained in the F\textsubscript{2} of gram-1 × prol-1 and phan-249 × prol-1, respectively, and their genotypes confirmed by PCR.

Molecular biology

cDNA clones of AmYAB2 (AY451398), AmFIL (GRAM, AY451396) and AmYAB5 (PROL, AY451397) were obtained by low-stringency screening of an Antirrhinum inflorescence cDNA library with the Arabidopsis INNER NO OUTER gene (Villanueva et al., 1999). Additional cDNAs from these genes and two additional paralogues, AmCRC (AY451399) and AmNO (AY451400) were kindly provided by Zsuzsanna Schwarz-Sommer (MPIZ, Germany). The introns of AMYAB3 and AMYAB5 were identified by PCR amplification of genomic DNA. Sequence phylogenies were reconstructed from inferred full-length amino acid sequences using CLUSTAL and PAUP software.

Both gram-1 and gram-3 gave rise to a low frequency of wild-type progeny and gram-3 produced wild-type branches, consistent with both mutations being caused by unstable transposons. Transposons were identified by PCR with transposon- and GRAM-specific primers. Primers to a sequence conserved in CACTA transposons were used with AMYAB5-specific primers to screen DNA from a collection of mutants maintained at IPK, Gatersleben. These detected a CACTA insertion within the first intron of AmYAB5 in the inbred line, MAM265, which had slightly larger leaves than the wild-type lines, JI.75 and Sippe 50. In an F\textsubscript{2} of MAM265 × JI.75 (n=94) leaf size showed continuous variation and Student’s t-tests detected no significant differences in leaf length or width between amyb5/amyab5, amyb5/+ and homozygous wild-type siblings (P>0.20 in all pair-wise comparisons). This suggested that the amyb5 allele did not condition a mutant phenotype and that the phenotype of MAM265 was consistent with a different genetic background to JI.75 or Sippe 50. The amyb5 allele, however, segregated with an enhanced gram mutant phenotype in ~6% of the F\textsubscript{2} progeny of MAM265 × gram-1. These plants (n=23) were confirmed as amyb5 gram-1 double mutants by PCR genotyping, while all 18 tested gram mutant siblings carried at least one wild-type AmYAB5 allele, indicating enhancement of the gram phenotype by amyb5, or a very closely linked gene. Two amyb5 gram double mutants produced branches with a gram single mutant phenotype. PCR analysis confirmed that these branches carried revertant AmYAB5 alleles with sequence footprints characteristic of CACTA transposon excision;
strongly suggesting that enhancement of the *gram* phenotype was due to the *amyab*\textsuperscript{5} mutation. In the absence of other detectable mutations in MAM265, which had originally been proposed to carry the *prolongata* (*prol*) mutation, the *amyab*\textsuperscript{5} allele was named *prol*\textsuperscript{1}.

Three PHB homologues, most similar to PHB, REV and *ATH-B8/ATH-15*, respectively, were obtained by probing an *Antirrhinum* cDNA library with a PHB cDNA. The most PHB-like gene (*AmPHB*; \texttt{AY451395}) encoded a protein with 84\% identical amino acids to PHB in a 230 amino acid region spanning the START domain.

**Microscopy**

Epidermal impressions were made in Locite Superglue on a microscope slide and examined with phase contrast optics. Histological sections (5 \textmu m) were made from material embedded in JB-4 resin and stained with Toluidine Blue (Ruzin, 1999). Scanning electron microscopy and in situ hybridisation were performed as described previously (Golz et al., 2002) The digoxigenin-labelled probes *GRAM*-long and *PROL* were transcribed from near full-length cDNA clones and the *GRAM* 3\' probe from the final three exons downstream of the Tn3 insertion in *gram-3*. Antisense *AmPHB* probes were transcribed from a 700 bp cDNA that spanned the region encoding the START domain.

**Results**

**GRAM is needed for adaxial-abaxial asymmetry and growth of lateral organs**

The recessive *gram*\textsuperscript{-3} mutation was identified in a transposon mutagenesis screen for altered leaf growth and shown to be allelic to two classic mutations, *gram*\textsuperscript{-1} and *gram*\textsuperscript{-2}. All three mutations had similar developmental effects.

Wild-type leaves differ in size according to the node at which they are produced, reaching their maximum mature length and width at nodes 3 or 4 (Fig. 1A). All leaves of *gram*\textsuperscript{-1} and *gram*\textsuperscript{-2} mutants were consistently half the width of wild-type ones (Fig. 1A,B) and also shorter than wild type up to node 3, after which they were similar in length.

The adaxial epidermis of a wild-type *Antirrhinum* leaf consists of large irregular pavement cells with infrequent hairs and stomata (Fig. 1C), the abaxial epidermis comprises small pavement cells, fewer hairs but frequent stomata and cells at the leaf edge are domed and elongated. Internally, palisade mesophyll is found adaxial to spongy mesophyll and the junction between these tissues runs to the leaf edge (Fig. 1E). The lower chlorophyll content and larger air-spaces of the spongy mesophyll make the abaxial side of the leaf appear light green (Fig. 1B).

Leaves of *gram* mutants have regions of darker green tissue, characteristic of the adaxial side of the leaf, at the abaxial margins (Fig. 1B). In section, elongated palisade cells extend around the edge of the leaf into the abaxial margin (Fig. 1F), making the lamina thicker towards its edge. This phenotype

![Image of leaf structures](https://example.com/leaf_structures.png)
suggested that GRAM is needed for abaxial cell identity at the leaf margin and that adaxial identity occurs in its absence. Similarly, epidermal cells with adaxial characters were found in the abaxial margins and the cells normally associated with the leaf edge extended further into the abaxial epidermis (Fig. 1D), suggesting that GRAM also promotes abaxial identity in epidermal cells. Epidermal marginal cells, which normally form at the leaf edge overlying the junction between spongy and palisade mesophyll, were absent from the displaced adaxial-abaxial boundary in gram leaves (Fig. 1B,D).

Loss of GRAM activity also caused adaxial mesophyll cells away from the leaf margin to partly resemble abaxial spongy mesophyll in shape and spacing (Fig. 1F). This suggested that GRAM is not only needed for abaxial identity at leaf margins but to promote adaxial identity elsewhere in the leaf. More severe loss of adaxial cell identity was observed occasionally in needle-like leaves produced by gram-3 mutants (Fig. 1G), which contained a central vein in which xylem was surrounded by phloem (Fig. 1H,I). Because phloem develops abaxial to xylem in the wild-type leaf, the needle-like leaves appeared to have lost adaxial, and gained abaxial, identity. gram mutant petals, like leaves, were smaller than wild-type ones and free for more of their length (Fig. 2A,E), suggesting that GRAM is also needed for petal growth. Where petals remained united, pronounced furrows developed in their adaxial (inner) sides flanked by ridges (arrowheads in Fig. 2F,G). Cells within the furrow had ectopic abaxial identity, as seen by their darker red pigmentation and lack of yellow hairs. Similarly, the ridges flanking each furrow contained a radially symmetric vein with an abaxial arrangement of cell types (compare Fig. 2B-D with Fig. 2F-H), suggesting that GRAM is needed for adaxial identity at petal margins. gram mutants also showed reduced growth of the style and occasional homeotic conversions of floral organ identity (Navarro et al., 2004).

**GRAM promotes growth in a marginal domain of leaf primordia**

Although mature leaves of gram mutants were narrower than those of the wild type, they originated from primordia of similar size (data not shown), suggesting that the reduced width of gram leaves was a consequence of less growth after initiation. gram mutant leaves contained ~50% fewer cells in the lateral axis when compared to a wild-type leaf at the same node (Fig. 1), suggesting that reduced cell divisions were involved in the reduced leaf width. To test whether reduced cell division occurred throughout the developing leaf, or whether it was largely confined to a particular region, we analysed the contributions of different cell layers to wild-type and gram mutant leaves.

In Antirrhinum, in common with most dicots, the SAM consists of three cell populations – a single layer of protoderm cells (L1), a single layer of sub-epidermal cells (L2) and a core of L3 cells. The fates of cells derived from the L2 layer were followed in GRAM+ and gram mutant leaves using stable periclinal chimeras in which L2 was marked by an olive mutation that reduces chlorophyll content (see Materials and methods). Towards the midrib of GRAM+ leaves, L2 contributed one layer of yellow adaxial palisade cells and one abaxial layer of yellow spongy mesophyll cells covering a core of L3-derived green cells (Fig. 3A). The medial part of the leaf therefore appeared green. Nearer the leaf edges, all internal cells were derived from L2 and therefore the margins appeared yellow. The proportion of the blade with internal L2-derived cells varied from about one-third to two-thirds of the leaf width (Fig. 3A). The boundary between green (L3-derived) and yellow (L2-derived) tissue did not correspond to any structural feature and its position varied in different leaves or in opposite halves of the same leaf. In contrast, L3 contributed most of the internal cells in a gram mutant leaf (often more cells than in GRAM+) and the position of the boundary between yellow and green tissue was more consistent (Fig. 3B). This suggested firstly, that GRAM promotes cell divisions in the margins of leaf primordia, where internal tissues are derived entirely from L2, and secondly that L3 contributes more cells to the gram

![Fig. 2. gram mutations affect growth and asymmetry of petals.](image)
GRAMINIFOLIA promotes leaf growth and polarity

To test when in development GRAM activity was required for localised cell proliferation, the expression of CYCLIN D3a RNA, which correlates with cell division rates in organ primordia (Gaudin et al., 2000), was compared in gram and wild-type leaves. Expression in newly initiated wild-type primordia was uniform and then became concentrated in the growing margins, remaining detectable there until at least stage P5 (Fig. 3C). gram mutant primordia resembled those of wild type in size early in development and showed a similar early pattern of CYCLIN D3a expression (Fig. 3D). However, from stage P3 or P4, expression spread from the margins to more central cells and persisted there until at least stage P7, consistent with a shift in growth from a marginal to more central region of the leaf later in development.

GRAM encodes YABBY activity

Both gram-1 and gram-3 showed the genetic instability characteristic of transposon-induced mutations. Because GRAM was needed to promote abaxial organ identity and a similar role had been attributed to members of the YAB gene family in Arabidopsis (Siegfried et al., 1999), we tested whether GRAM might encode YAB activity. Five Antirrhinum YAB genes were identified as cDNAs, each encoding a protein with the N-terminal zinc finger and C-terminal HMG-like YAB domain characteristic of the family (Fig. 4B).

Phylogenetic analysis placed one of these proteins, termed AmFIL, in a well-supported clade with the products of the Arabidopsis FILAMENTOUS FLOWER (FIL) and YAB3 genes (Fig. 4A). A second Antirrhinum protein, AmYAB5, appeared orthologous to YAB5. Amplification from genomic DNA identified six introns in AmFIL and AmYAB5 in positions that were conserved between Antirrhinum genes and with their Arabidopsis homologues (Fig. 4B). An AmFIL probe detected a different RFLP in each gram mutant that segregated with the gram mutant allele (data not shown). Sequence analysis revealed that the RFLPs were caused by transposon insertions; gram-3 carried a copy of the Tam3 transposon in exon 5 of AmFIL and gram-1 and gram-2 carried Tam2 in introns 4 or 5, respectively (Fig. 4B). The gram-3 allele had the potential to encode a protein in which the C-terminal part of the highly conserved YAB domain was replaced with 69 amino acids encoded by Tam3.

Tam3 was lost from gram-3 in five independent GRAM+ revertants. None carried sequence footprints, often associated with Tam3 excision, presumably because footprints would disrupt the highly conserved YAB domain. A single reversion of gram-1 to wild-type involved the loss of Tam2 together with 85 bp of flanking intron sequence. These results confirmed that AmFIL corresponded to the GRAM locus.

A transposon insertion in the AmYAB5 gene was also identified in an inbred line carrying the classic mutation, prolongata-1 (prol-1; Fig. 4B) (Stubbe, 1966), but conditioned no mutant phenotype in an otherwise wild-type genetic background (see Materials and methods).

Fig. 3. GRAM promotes marginal leaf growth. (A) The leaves of periclinal chimeras in which L2-derived cells carry an oli mutation that reduces chlorophyll content. L2-derived cells contribute a variable proportion of the internal cells of the leaf – seen as a yellow marginal region in the surface view (above) or as cells showing no red chlorophyll auto-fluorescence under UV light in section (below). (B) The loss of L2-derived tissues at the leaf margins of gram mutants is partially compensated for by an increased growth of the L3 layer. (C,D) Transverse sections of wild-type (C) and gram (D) vegetative apices probed with the CYCLIND3a probe. Note the shift in CYCLIN expression to more internal regions of gram mutant leaves.

Fig. 4. Structure and evolution of GRAM and PROL. (A) A neighbour-joining tree showing the relative similarity of the full-length Antirrhinum and Arabidopsis YABBY proteins, suggesting their evolutionary relationships. Bootstrap values (1000 replicates) are given. (B) The structure of the GRAM and PROL loci and mutant alleles. Boxes represent exons (black are translated, white are untranslated). The regions encoding the N-terminal zinc finger domain and the C-terminal YAB domain are stippled. Transposon insertions are shown as triangles (not to scale); numbers denote Tam2 or Tam3.
**GRAM and PROL are expressed abaxially in developing lateral organs**

GRAM RNA expression, revealed by in situ hybridisation, was similar in all lateral organs. It was detected first in incipient primordia within the SAM or floral meristem (stage P0 in leaves) and abaxially in newly initiated (early P1) primordia (Fig. 5A,B). It then became restricted mainly to the abaxial margins of growing primordia from about stage P2 (Fig. 5B). This later pattern of expression was consistent with the proposed role of GRAM in promoting abaxial cell fate and growth in leaf margins. PROL RNA was always less abundant than GRAM (Fig. 5D,E), but like GRAM it was expressed abaxially from stage P1. Later expression, unlike GRAM, was detected predominantly in provascular cells and to a lesser extent in the mesophyll cells in the centre of each primordium. PROL RNA was not detectable in the prol-1 mutant by in situ hybridisation or RT-PCR (data not shown).

**GRAM, PROL and PHAN promote adaxial organ fate**

GRAM and PROL RNA, which are expressed in abaxial cells of wild-type leaf primordia, were absent from the margins of gram mutant primordia (Fig. 5C,F), consistent with the loss of abaxial identity from this region. GRAM expression was unaffected by the prol-1 mutation that has no effect on leaf development (data not shown).

Loss of PHANTASTICA (PHAN) activity has the opposite effect to gram of allowing leaf cells in adaxial positions to assume abaxial fates (Waites and Hudson, 1995). The degree to which phan mutant leaves are abaxialised increases with decreasing temperature: at 25°C all leaves are mosaics of adaxial and ectopic abaxial tissue (Fig. 6C), at 20°C leaves at higher nodes on the plant are needle-like and consist only of abaxial cell types (Fig. 6A) while at 15°C phan mutants are unable to maintain a functional SAM.

Because GRAM is required for abaxial identity, ectopic abaxial fate in phan mutant leaves might result from ectopic GRAM expression. Consistent with this, the domain of GRAM expression was found to extend into the adaxial region of phan mutant primordia initiated at 20°C (Fig. 6LJ). If GRAM activity was responsible for ectopic abaxial identity, gram mutations would be expected to suppress the polarity defects of phan mutant leaves. However, gram mutations were found to enhance, rather than suppress the phan mutant phenotype, phan gram double mutant seedlings had more severely abaxialised cotyledons and differentiated cells in place of the SAM (Fig. 6E-H). Shoots eventually arose from adventitious meristems in the hypocotyl or the base of cotyledon petioles (Fig. 6B,D). Leaves produced from phan gram shoots were radially symmetrical and abaxialised, based on their histology, arrangement of vascular cells in the central vein (Fig. 6K,L) and ubiquitous expression of mutant gram RNA (Fig. 6M). Loss of GRAM activity also rendered phan mutants insensitive to temperature (Fig. 6A-D). Enhancement of the phan mutant phenotype by gram mutations suggested that GRAM promotes adaxial organ fate redundantly with PHAN. Because the leaves of phan gram double mutants retain abaxial identity, GRAM also appeared unnecessary for abaxial organ identity in the absence of adaxial fate specification.

To determine whether GRAM and PROL function redundantly to promote abaxial identity, plants were generated carrying both gram and prol-1 mutations. Surprisingly prol-1 enhanced the gram mutant phenotype in the same way as phan mutations (Fig. 6N-S). Initially all gram prol seedlings lacked a SAM, however shoots eventually formed from adventitious meristems at the root-hypocotyl junction (Fig. 6N). All of the leaves that formed on these shoots were radially symmetrical and had hairs that were specific to the abaxial surface of wild-type leaves (Fig. 6O-Q). Lack of adaxial cell types was confirmed by histology (Fig. 6R) and by the ubiquitous expression of gram transcript and complete absence of AmPHB expression (Fig. 6S,T). These results suggest that PROL promotes adaxial organ fate redundantly with GRAM and that it is not required, alone or redundantly with GRAM, for adaxial fate when adaxial fate is not specified. Unlike gram mutations, prol-1 did not modify the phan mutant phenotype (data not shown).

Neither GRAM nor PROL are needed for abaxial cell fate in the absence of adaxial identity. The role of GRAM in promoting abaxial organ fate might therefore be to repress adaxial identity. To test this we examined its interaction with AmPHB, an Antirrhinum homologue of PHB, which is necessary and
3667 GRAMINIFOLIA promotes leaf growth and polarity

sufficient for adaxial fate in Arabidopsis leaves (McConnell et al., 2001). Sense RNA from AmPHB, like its Arabidopsis homologue, was expressed in the wild-type SAM, uniformly in newly initiated leaf primordia and adaxially from late stage P1 (Fig. 5G,H). In contrast, AmPHB expression was not adaxially restricted in P2 and P3 primordia of gram mutants and was particularly abundant at their margins (Fig. 5I). This expression pattern was therefore consistent with GRAM acting to repress AmPHB expression and adaxial identity from at least stage P2.

GRAM acts non cell-autonomously

GRAM and PROL are expressed abaxially but required, non cell-autonomously, to promote the identity of adaxial cells. To test whether GRAM can also affect abaxial fate non cell-autonomously, we exploited the ability of the gram-3 mutation to give rise to clones of wild-type cells following transposon excision. Plants homozygous for the unstable gram-3 allele occasionally produced branches with a wild-type phenotype. In most cases, the flowers on these branches gave rise to ~75% wild-type progeny on self-pollination, suggesting that the subepidermal (L2) layer of the SAM, from which gametes are derived, carried a revertant GRAM+ allele. One phenotypically wild-type branch, however, produced only gram mutant progeny, suggesting that it was a periclinal chimera carrying a revertant GRAM+ allele in either the L1 or L3 layers of the SAM. These possibilities were tested by in situ hybridisation with a probe that could detect wild-type GRAM RNA but not the transcripts produced from gram-3, which terminate within the transposon insertion (Fig. 7A-D). In the chimeric wild-type branch, the downstream probe detected high levels of GRAM transcripts only in L1 cells within the normal, abaxial domain of GRAM expression (Fig. 7F), indicating that this branch had normal GRAM activity in epidermal cells but not in sub-epidermal, L2-derived cells. Consistent with this, a wild-type GRAM+ allele could be amplified from the revertant branch, but not from gram mutant branches of the same plant. These findings indicated that GRAM activity in abaxial epidermal cells is sufficient for normal identity and proliferation of more adaxial cells, presumably via an intercellular signal.

Discussion

We have shown that the YABBY transcription factor, GRAM, is needed for abaxial identity and growth at the margins of Antirrhinum leaves. This role correlates with GRAM expression in an abaxial domain of newly initiated organ primordia and its persistence in abaxial margins of the growing leaf.

Loss of GRAM activity allows cells in abaxial marginal positions to assume adaxial identities. Because the leaves of
plants lacking GRAM activity in a *phan* or *prol* mutant background retain abaxial identity. GRAM does not appear to be necessary for abaxial fate per se, but rather to exclude adaxial identity from the abaxial leaf margins. This does not exclude the possibility that other genes might specify abaxial identity independently of GRAM. Obvious candidates include other members of the *YAB* gene family, at least two of which – *PROL* and *AmYAB2* – are expressed abaxially in developing leaves of wild-type and abaxialised *phan gram* mutants (data not shown). *PROL*, however, is not needed for abaxial identity, alone or in combination with GRAM, because abaxial identity is retained in leaves lacking activity of both GRAM and *PROL* or *PHAN* and *PROL*. In *Arabidopsis*, members of the *KAN* family are also required for abaxial fate (Eshed et al., 2001; Kerstetter et al., 2001). Two *KAN* genes are known to be expressed in *Antirrhinum* leaves (J.F.G., unpublished) and might therefore specify abaxial fates in the absence of GRAM and PROL activity.

Activity of the HD-ZIP proteins PHB, PHV and REV is sufficient to confer abaxial identity in *Arabidopsis* leaves (Emery et al., 2003; McConnell et al., 2001) and restriction of their activity to an adaxial domain is considered to be an early step in elaboration of organ asymmetry. In *Antirrhinum* leaves, GRAM is needed to restrict expression of *AmPHB*, a *PHB* homologue, to an adaxial domain of organ primordia, consistent with GRAM acting to repress HD-ZIP-dependent adaxial fate. It is, however, unclear whether GRAM is needed to set up the domain of HD-ZIP expression or to maintain it. It is also unclear whether abaxial expression of GRAM is established in response to adaxial HD-ZIP activity.

The role of GRAM in repressing adaxial fate differs from that proposed for the homologous *Arabidopsis* genes, *FIL* and *YAB3*. Reduced activity of both *Arabidopsis* genes has a similar effect to *gram* mutations on leaf growth (Kumaran et al., 2002; Siegfried et al., 1999), suggesting that *FIL* and *YAB3* together provide a GRAM-like function. However fill *yab3* mutants have less severe polarity defects involving only a partial loss of abaxial cell characters but no clear gain of adaxial identity. In this respect the effects of *gram* mutations are more similar to loss of both *KAN1* and *KAN2* activity in *Arabidopsis*, which is also accompanied by ectopic HD-ZIP expression, as in *gram* (Eshed et al., 2001). The different requirements for GRAM compared to *FIL* and *YAB3* might reflect divergence in the function of the *YAB* gene family in the two species (e.g. from GRAM having assumed or retained *KAN*-like functions). Alternatively, they might result from different degrees of functional overlap between *YAB* genes within each species. These possibilities might be tested with additional *yab* loss-of-function mutations in both species. In the case of *Antirrhinum*, reduced activity of *PROL*, for which no orthologous *Arabidopsis* mutant has been reported, has no developmental effects when GRAM is active, suggesting that *PROL* is redundant. Because reduced activity of both GRAM and *PROL* results in loss of adaxial identity, it does not reveal whether *PROL* and GRAM might function redundantly to repress adaxial identity.

In addition to promoting abaxial fate by repression of adaxial identity, GRAM and *PROL* together promote adaxial identity. This role is apparent in the loss of adaxial cell characters from *gram* single mutant leaves and the complete replacement of adaxial by abaxial tissues in *gram prol* double mutant leaves. In *gram prol*, the polarity defect is also accompanied by reduced SAM activity, as seen in other mutants with abaxialised leaves (e.g. Eshed et al., 2001; Waites and Hudson, 2001). Although GRAM is expressed ectopically in the abaxialised leaves of *phan mutants*, *gram* mutations also enhance the abaxialised organ phenotype of the *phan* mutants in a similar way to the *handlebars* (*hb*) mutation (Waites and Hudson, 2001) and, like *hb*, remove its sensitivity to cold. This is consistent with *HB* and GRAM acting in a cold-sensitive pathway that promotes adaxial identity redundantly with *PHAN*. However, the relationship between these genes is likely to be more complex because *hb gram* double mutants (not shown) resemble *hb gram*, whereas the *prol* mutation enhances the phenotype of *gram*, but not *phan* mutants.

The finding that the *KNOX* gene, *HIRZ*, is expressed ectopically in abaxialised leaves of *phan* mutants has lead to the suggestion that *KNOX* expression might cause polarity defects (e.g. Tsianastis et al., 1999). In *Arabidopsis*, *FIL* and *YAB3* have also been found necessary to prevent *KNOX* expression in leaves (Kumaran et al., 2002), suggesting that GRAM might have a similar role in *KNOX* repression and that the enhanced mutant phenotype of the *phan gram* mutant might reflect increased *KNOX* mis-expression. Two observations,
however, argue against this. Firstly, ectopic KNOX expression could not be detected in gram single mutant leaves and secondly, a Hirz gain-of-function mutation that causes ectopic HIRZ expression, as in phan mutants, failed to cause leaf polarity defects in a GRAM \* background or to enhance the polarity defects of gram mutants (data not shown) (Golz et al., 2002).

Because both GRAM and PROL show abaxially restricted expression but promote adaxial identity, they appear to be necessary for a non cell-autonomous signal from the abaxial to adaxial domain. A further non cell-autonomous role of GRAM was revealed by a periclinal chimera in which GRAM expression only in the most abaxial cell layer (L1) of the primordium was sufficient for normal development of leaves and flowers. The simplest explanation for both these non cell-autonomous effects is that they involve the same intercellular signalling mechanism. Because GRAM protein is absent from the adaxial region of developing leaves (Navarro et al., 2004), it is likely to regulate production of a downstream signal in abaxial cells, rather than to acts as a signal itself.

Loss of adaxial identity is also observed in plants lacking activity of GRAM and STYLOSA (STY) (Navarro et al., 2004), suggesting that STY is also required for the adaxial promoting signal. GRAM and STY proteins interact physically and are co-expressed only in abaxial cells of early organ primordia, suggesting that STY and GRAM together regulate the signalling mechanism from early in organ development.

GRAM has opposite roles in the two parts of the leaf – repression of adaxial identity in the abaxial domain and promotion of adaxial identity. This seems unlikely to result from differences in the concentration of a signalling molecule, because the boundary of GRAM expression can be shifted abaxially to the junction between L1-L2 cell layers in a periclinal chimera without causing a shift in the boundary between development of adaxial and abaxial tissues.

Although the adaxial-promoting and adaxial-repressing roles of GRAM might appear paradoxical, similar phenomena appear to be common in other signal-response systems. For example, the Decapentaplegic signalling protein is secreted by the most dorsal cells of the Drosophila embryo and promotes expression of the Zerkn"ult (Zen) transcription factor, which confers amnioserosa fate, in a dorsal domain (Ray et al., 1991). It also induces more ventral expression of Brinker (Br), which represses Zen transcription cell-autonomously (Jazwinska et al., 1999). The interaction of Br with Zen is necessary to refine the dorsoventral boundary of Zen expression (Muller et al., 2003). In an analogous way the opposite effects of GRAM might serve to refine the boundary between adaxial and abaxial cells of organ primordia preventing the specification of intermediate cell identities. It might also serve to maintain the boundary, which is proposed to be necessary for lateral growth. This view of GRAM function is consistent with the observed loss of ad-abaxial distinction at the margins of gram leaves and the loss of lateral growth in this region. Use of CYCLIN D3a expression as a marker for cell division, suggested that lateral growth of primordia was not affected until relatively late in development, consistent with a requirement for GRAM to maintain an ad-abaxial boundary. Both adaxial asymmetry and growth are maintained in the medial parts of the leaf, perhaps because of the activity of other genes (e.g., additional YAB family members). A requirement for GRAM to maintain a growth-promoting ad-abaxial boundary is also consistent with the lack of ectopic growth at the ectopic boundary between adaxial and abaxial cell types in the ventral margin of gram mutant leaves.

Evidence for ad-abaxial signalling in leaves has also been provided by Arabidopsis plants with reduced activity of the abaxially expressed KAN1 gene. kani mutants show abaxial defects, but also dosage-dependent reductions in adaxial trichome density (Kerstetter et al., 2001).

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