Molecular and genetic interactions between *STYLOSA* and *GRAMINIFOLIA* in the control of *Antirrhinum* vegetative and reproductive development

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Summary

***STYLOSA* (*STY*) in *Antirrhinum* and *LEUNIG* (*LUG*) in *Arabidopsis* control the spatially correct expression of homeotic functions involved in the control of floral organ identity. We show here that the *sty* mutant also displays alteration in leaf venation patterns and hypersensitivity towards auxin and polar auxin transport inhibitors, demonstrating that *STY* has a more general role in plant development. *STY* and *LUG* are shown to be orthologues that encode proteins with structural relation to GRO/TUP1-like co-repressors. Using a yeast-based screen we found that STY interacts with several transcription factors, suggesting that STY, like GRO/TUP1, forms complexes in vivo. Proteins of the YABBY family, characterised by containing a partial HMG domain, represent a major group of such interactors. In vivo association of STY with one of the YABBY proteins, GRAMINIFOLIA (GRAM), is supported by enhanced phenotypic defects in *sty gram* double mutants, for instance in the control of phyllotaxis, floral homeotic functions and organ polarity. Accordingly, the *STY* and *GRAM* protein and mRNA expression patterns overlap in emerging lateral organ primordia. *STY* is expressed in all meristems and later becomes confined to the adaxial domain and (pro)-vascular tissue. This pattern is similar to genes that promote adaxial identity, and, indeed, *STY* expression follows, although does not control, adaxial fate. We discuss the complex roles of *STY* and *GRAM* proteins in reproductive and vegetative development, performed in part in physical association but also independently.**

Key words: GRO/TUP1, Co-repressor, Floral organ identity, Leaf development, Auxin

Introduction

Flowers of angiosperms are typically composed of four structurally and functionally distinct organs, named sepals, petals, stamens and carpels, which develop sequentially and are organised in four whorls. Although the number and shape of these organs varies among species, the genetic control of organ identity is conserved; the B-function controls petal identity, the C-function identifies carpels, the overlap of B and C results in stamens and the absence of B and C in sepals (Coen and Meyerowitz, 1991; Schwarz-Sommer et al., 1990). The spatial control of the C-domain is mediated by the complex A-function. This complexity is indicated by the large number of class A mutants in *Arabidopsis* (reviewed by Lohmann and Weigel, 2002) and by the fact that these genes have additional functions in the control of floral meristem identity, organ growth or various aspects of carpel and ovule development (Byzova et al., 1999; Krizek et al., 2000; Liu et al., 2000). Furthermore, in spite of the similarity of the B- and C-functions across the plant kingdom, the mechanism of restriction of the C-domain appears to differ in *Arabidopsis* and *Antirrhinum* (reviewed by Schwarz-Sommer et al., 2003). It is therefore of interest to determine how the function of genes involved in the control of C compares between these species.

The *Antirrhinum* mutants *stylosa* (*sty*), *fistulata* (*fis*), *choripetala* (*cho*) and *despenteado* (*des*) display partial loss of control over the establishment or maintenance of the outer expression domain of the B- and C-functions in their flowers (McSteen et al., 1998; Motte et al., 1998; Wilkinson et al., 2000). This is revealed by petaloid sepals in the first floral whorl and/or by stamenoid features in the second whorl as a result of ectopic expression of class B and class C genes. Interestingly, ectopic expansion of the B and C functions often occurs concomitantly suggesting that the regulation of their expression may involve common factors. In addition, the mutants display other abnormal features such as narrow vegetative and floral organs (in *cho* and *des*), or fasciation and aberrant carpels (in *sty*). The homeotic defects in the single mutants are not striking, although they can become more pronounced depending on the genetic background. Double mutant combinations, however, display severely enhanced homeotic phenotypes in all genetic backgrounds. This suggests that STY, FIS, CHO, DES and some additional factors function

together, perhaps as components of a larger protein complex, or in independent pathways that converge to control the outer limits of the B and C domains.

In this report we show that *STYLOSA* (*STY*) is the orthologue of *LEUNIG* (*LUG*), an *Arabidopsis* gene that represses the C-function gene *AGAMOUS* in the two outer whorls (Conner and Liu, 2000; Liu and Meyerowitz, 1995). The STY and LUG proteins are structurally related to GRO/TUP1-like co-repressors found in all metazoans and yeasts (Conner and Liu, 2000). GRO/TUP1 interact with diverse DNA-binding partners and are involved in regulation of a broad range of developmental processes (reviewed by Fisher and Caudy, 1998). One such partner in *Drosophila*, mammals and yeast is represented by a heterogeneous group of proteins that contain a DNA-binding HMG box (Brantjes et al., 2001; Cavallo et al., 1998; Deckert et al., 1995). This association appears to be important for the formation of larger nucleoprotein complexes, termed 'repressosomes', where HMG-box proteins represent architectural factors (Courey and Jia, 2001). We found that STY interacts in yeast with GRAMINIFOLIA (GRAM), a member of the plant-specific YABBY protein family (Golz et al., 2004). YABBY proteins have a highly conserved N-terminal zinc-finger domain and a truncated HMG domain (the YABBY domain), whereas the internal region between these domains and the C terminus are variable (Bowman and Smyth, 1999; Sawa et al., 1999b). In vitro DNA-binding studies with the YABBY protein FILAMENTOUS FLOWER (FIL) showed that the HMG box is essential for protein-DNA interaction and the zinc-finger domain stabilises the protein structure (Kanaya et al., 2002).

GRAM together with other YABBY proteins such as PROLONGATA (PROL) is involved in the control of leaf polarity and growth. In addition, more severe *gram* mutants also display mild homeotic conversions indicating a role of *GRAM* in the control of expression domains of the B and C functions. Genetic interactions between *sty* and *gram* mutants revealed common and distinct functions during vegetative and reproductive development, one aspect of which is the co-operative control of the B and C domains. We also report on an unexpected connection between *STY* and hormone-mediated processes, suggesting a more general role for STY in developmental control.

Materials and methods

Plant material and genetic stocks

Plants were grown in the greenhouse at a daytime temperature of 18-25°C and with additional light during the winter. For growth at 17°C 3-week-old seedlings were transferred to a climate chamber and cultivated under standard conditions (16 hours light and 8 hours dark).

The wild-type lines JI98 (the progenitor of line 165E), JI75 and the *gram-3* mutant (Golz et al., 2004) were kindly provided by Rosemary Carpenter (John Innes Centre, Norwich, UK). The wild-type line Sippe 50 and the mutants *sty*, *phan*-ambigua and *gram-1* (referred to as *phan* and *gram*, respectively) were obtained from the collection at the IPK, Gatersleben, Germany (accession numbers MAM428, MAM316, MAM250 and MAM 146, respectively). To reduce the influence of the genetic background the genuine 'Gatersleben' background, corresponding to the Sippe 50 line was generally used. The 165E line was used for segregation analyses to enhance the probability of sequence polymorphisms between mutant and wild-type alleles.

Arabidopsis *lug*-1 seeds (N8031) were obtained from the Nottingham Stock Centre.

Molecular biology

Detailed information on isolation of proteins, nucleic acids, PCR primers, PCR conditions and other methods used but not explicitly documented in this report are available upon request.

DNA- and RNA-related methods

DNA for large scale segregation studies by PCR was prepared from 50-100 mg of leaves, adopting a protocol developed for *Arabidopsis* (Klimyuk et al., 1993) using 96-well plates. Polymorphisms were detected as CAPS (cleaved amplified polymorphic sequences) by restriction of PCR fragments and separation on agarose gels or as single-nucleotide polymorphisms (SNP) with the WAVE method (Kuklin et al., 1997).

Protein-related methods

The cDNAs of the entire GRAM protein and amino acids 173-509 of STY (displaying the lowest degree of homology between STY and STY-L; see Fig. 1) were cloned into the pGEX-3X and pQE60 vectors, respectively. The recombinant proteins were expressed in *E. coli* and purified by utilising the N-terminal GST extension (for GRAM) or the C-terminal His-tag (for STY). Antisera were produced in rabbits (Pineda Antibody Service, Berlin, Germany) and affinity purified, in two steps, against antigens immobilised on HiTRAP NHS-activated HP columns (Amersham Biosciences). First, most of the antibodies interacting with the tags and nonspecific antibodies cross-reacting with plant proteins were removed. In the second step antibodies specifically interacting with the immobilised GRAM or STY antigens were obtained.

Specificity of the antibodies was tested in western blots with nuclear proteins prepared from wild-type and mutant plants (not shown). The purified STY antibody detected a single protein of 130 kDa size that was not expressed in the *sty* mutant. The purified GRAM antibody detected several proteins of similar size (approximately 20-30 kDa) in the wild type, the most abundant of which was absent in *gram-3* tissues. In spite of this ambiguity, no cross-reaction was detectable in *gram-3* mutant sections in immunolocalisation experiments (see Results).

Yeast two-hybrid screening

The coding region of the *STY* cDNA was cloned into pGBT9 and into pBKT7. The screening procedure after library transformation followed a published protocol (Davies et al., 1996). For detecting ternary complexes the AmSEU3A cDNA was cloned into the TFT vector and used as previously reported (Egea-Cortines et al., 1999). Some of the screens were performed by applying the Matchmaker library construction and screening protocol (Clontech) and used a normalised full plant yeast expression library for mating (S. Masiero, Z.S.-S. and H. Sommer, unpublished). For directly testing interactions in yeast, cDNAs were cloned into pBKT7 and pGAD424.

In situ analysis of RNA and protein expression

Tissue preparation, in situ hybridisation and immunolocalisation experiments were performed as previously described (Davies et al., 1996; Perbal et al., 1996; Zachgo et al., 1995). The digoxigenin-labelled STY antisense probe contained the internal, non-redundant region of the STY cDNA (position 520 to 1520). The GRAM probe was prepared from the full-size GRAM cDNA.

Histology and scanning electron microscopy

Histological sections were prepared and viewed according to the method of Golz et al. (Golz et al., 2004). For observations on the vascular skeleton leaves were dehydrated in ethanol, cleared with NaOH and stained with basic fuchsin (Sigma) as described previously (Fuchs, 1963). Photographs were taken with a Leica MZ FIII

microscope using UV light. Scanning electron microscopy (SEM) with fresh freeze-fractured leaves was performed as reported previously (Efremova et al., 2001).

Auxin response and polar auxin transport inhibition assays

Three-week-old in vitro cultured seedlings (Heidmann et al., 1998) were transferred to 0.5× MS medium (Murashige and Skoog, 1962) containing polar auxin transport inhibitors or auxins. For inhibition of polar auxin transport 0.5-20 μM 1-*N*-naphthylphthalamic acid and 2,3,5-triiodobenzoic acid (NPA and TIBA, respectively, both from Duchefa Biochemie BV, Holland) were used as described previously (Mattsson et al., 1999). For auxin response assays, indoleacetic acid and 2,4-dichlorophenoxyacetic acid (IAA and 2,4-D, respectively, both from Sigma) were dissolved in 1 M NaOH and in DMSO, respectively, and were added at 0.5-6 μM to plant growth media.

For measurement of polar auxin transport (Okada et al., 1991) the upper end of 2.5 cm long inflorescence stem segments, adjacent to the oldest flower of 8-weeks-old plants, were submerged in 0.5× MS medium containing 1.45 μM IAA and 4.8 nCi/30 μl [^{3}H]IAA (Amersham). After incubation for 16 hours, the opposite 5 mm end of the segments was excised, the radioactivity extracted for 12 hours in 1 ml ethanol and measured in a Beckmann LS-6500 liquid scintillation counter. Segments with the basal end submerged (with movement in the physiological direction) were used as controls.

Results

Molecular cloning of *STYLOSA* by a candidate gene approach

In an effort to identify *Antirrhinum* genes involved in restricting the C function to the inner whorls of the flower, we reasoned that they might encode homologues of *Arabidopsis* genes known to control this process. A BLAST search with the amino acid sequence of LUG (Conner and Liu, 2000) in the *Antirrhinum* EST collection identified a contig composed of several cDNAs, tentatively named *AmLUG*. The longest of these was used to probe Southern blots with genomic DNA prepared from groups of mutant and homozygote wild-type plants of F_2 populations segregating for *sty*, *fis* or *cho* mutants. Restriction site polymorphisms were detected in *sty* mutant groups because of differences between intron sequences of the *AmLUG* allele present in *sty* (and in its progenitor allele in Sippe 50) and the allele present in the unrelated 165E line (the wild-type parent of the segregating population). The polymorphism was converted into a CAPS marker to follow the alleles in a large segregating population. Analyses of several hundred individual F_2 plants showed that the *AmLUG* allele present in the *sty* background could not be separated from the *sty* mutation by recombination. A single nucleotide deletion at position 418 of the *AmLUG* coding sequence was identified in the cDNA from *sty* plants, but not in cDNAs derived from Sippe 50 or other wild-type backgrounds. This change results in a frameshift that is predicted to cause an early termination of translation. Since no other changes were found in either the genomic or cDNA sequences of the progenitor wild-type line Sippe 50 or *sty*, we assume that *AmLUG* corresponds to *STY* (EMBL accession AJ620905).

RT-PCR or northern blot analysis revealed that the *sty* transcript in *sty* mutants is reduced by up to 90% when compared to wild type (not shown), possibly because of an instability of the mutant mRNA.

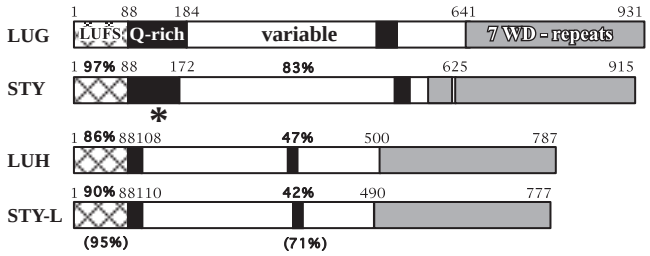


Fig. 1. Domain structure of GRO/TUP1-like co-repressors in *Arabidopsis* (LUG and LUH) and *Antirrhinum* (STY and STY-L). Individual domains are shown in boxes with different shading and domain designations are indicated for the LUG protein (Conner and Liu, 2000). Numbers show the position of amino acids within the proteins. Italic numbers indicate the percentage similarities relative to LUG domains (percentage similarity between LUH and STY-L in brackets). Asterisk indicates the position of the mutation in the *sty* allele. The sizes of domains are approximately to the scale.

GRO/TUP1-like co-repressors in *Antirrhinum*

There is a high degree of amino acid sequence conservation between LUG and STY with both proteins having an N-terminal LUFS domain, followed by a glutamine-rich domain, a variable region and a C-terminal 7 WD repeat domain (Fig. 1) (Conner and Liu, 2000). STY differs from LUG in having slightly shorter glutamine-rich domains and an additional WD repeat. This domain structure is similar to GRO/TUP1-like proteins in *Drosophila*, mammals and yeasts (Conner and Liu, 2000).

A second *STY-like* (*STY-L*; EMBL accession AJ620906) cDNA was identified in the *Antirrhinum* EST collection. *STY-L* appears to be the orthologue of *LUH* in *Arabidopsis* and displays a similar degree of amino acid sequence divergence from STY as that reported between LUG and LUH (Conner and Liu, 2000). These include a large deletion within the N-terminal glutamine-rich domain (Fig. 1) and conservation of amino acids that distinguish LUH from LUG (not shown). The functional consequences of these deviations are not known, but our studies in yeast suggest that STY and STY-L differ slightly in the range of proteins with which they can interact (Table 1, and not shown). Similarly, two structurally closely related TUP1-like proteins differing by internal deletions are present in fission yeast and play partially redundant roles in transcriptional regulation (Hirota et al., 2003; Janoo et al., 2001). In support of such redundancy, interaction between STY and STY-L can be observed in yeast (Table 1).

Searching for protein partners that interact with STY in yeast

We used a yeast two-hybrid screen to identify proteins that interact with STY expecting that such interactions might provide insight into the function of STY. The rationale behind this approach was that the role of GRO/TUP1-like proteins in transcriptional control arises from physical interactions with DNA-binding proteins (Flores-Saaib and Courey, 2000) and from interaction with the basal transcriptional machinery (Gromöller and Lehming, 2000; Zhang and Emmons, 2002).

Using STY as bait, several transcription factors were identified from a screen of about 5×10^7 yeast recombinants in various two-hybrid screens (Table 1; see Materials and methods). A major group of interactors included four proteins

Table 1. Protein partners of STYLOSA identified in yeast two-hybrid screens

Protein partner	*Arabidopsis* accession no.	*Antirrhinum* accession no.	Observed number	STY-L bait
GRAM	At2g45190	AY451396	47	yes
PROL	At2g26580	AY451397	1	yes
AmINO	At1g23420	AY451400	5	yes
AmYAB2	At1g08465	AY451398	†	no
AmCRC	At1g69180	AY451399	2‡	yes
AmSEU1	At5g62090	AJ620907	16	yes
AmSEU2	At5g62090	AJ620908	9	nt
AmSEU3A*	At1g43850	AJ620909	11	weak
AmSEU3B*	At1g43850	AJ620910	4	nt
STY-L	At2g32700	AJ620906	3‡	nt

*Two highly similar proteins encoded by two linked genes.
†Weak interaction detected after cloning of the respective protein as the prey.
‡Interaction in a yeast three-hybrid assay with STY as the bait and AmSEU3A as the ternary factor.
nt, not tested.

with sequence similarity to SEUSS (SEU) in *Arabidopsis*, which we called AmSEU. SEU is a putative co-repressor that interacts both genetically and physically with LUG (Franks et al., 2002). Our studies in yeast suggest that interaction between STY and AmSEU facilitates formation of higher order complexes with other proteins (Table 1).

A second major group of proteins that interact with STY in yeast belong to the YABBY family of transcription factors (GRAM, PROL and AmINO; see Table 1). *YABBY* genes were first identified in *Arabidopsis* (Sawa et al., 1999b; Siegfried et al., 1999) and form a small gene family of six members. In *Antirrhinum*, there are only five *YABBY* genes, with *GRAM* being the only orthologue of two closely related genes *FILAMENTOUS FLOWER* (*FIL*) and *YAB3* (Golz et al., 2004). Two other YABBY proteins, AmYAB2 and AmCRC, do not interact with STY, but AmCRC can form a higher order complex with STY and AmSEU (Table 1). In agreement with their structural and functional similarity to the *Antirrhinum* proteins STY and GRAM we found that the *Arabidopsis* protein LUG interacts with FIL and YAB3 in yeast.

The YABBY proteins identified in yeast screens all contained the N-terminal zinc-finger and the internal variable region, but in many instances lacked the YABBY-domain and the C-terminal region. This suggests that the internal variable region or the zinc-finger domain represent the region interacting with STY.

Based on the synergistic genetic interaction between *sty* and either *cho*, *fis* or *des*, we expected that some of the STY-interactors might be the proteins encoded by *FIS*, *CHO* or *DES*. However, CAPS markers developed for the four *AmSEU* genes and *STY-L* did not co-segregate with *cho*, *fis* or *des*.

Genetic interaction between *STY* and *GRAM* in the control of flower development

sty gram double mutants were generated and their phenotypes compared to the single mutant lines to test possible interactions in vivo.

The subtle homeotic defects of *gram* (Golz et al., 2004) (Fig. 2B,E) and *sty* flowers (Motte et al., 1998) (Fig. 2C,F) are dramatically enhanced in the *sty gram* double mutant (Fig. 2G-I). The whorled organisation of *sty gram* flowers is often disrupted, making it difficult to assign floral organs to a particular whorl (Fig. 2H). Most often the dorsal and the two ventral sepals in the first whorl are petaloid and second whorl organs are narrow, radialised or stamenoid (Fig. 2H). Stamens

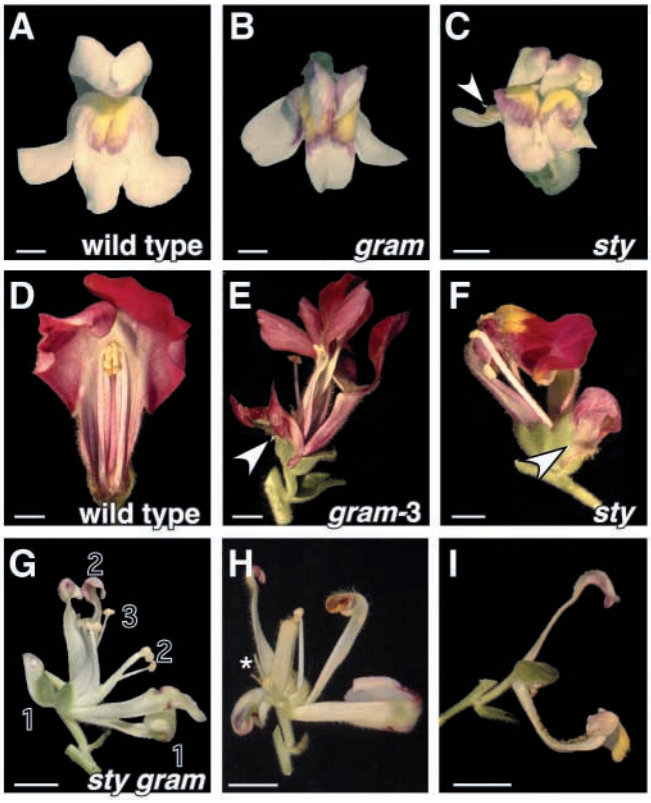


Fig. 2. Morphology of wild-type (A,D), *gram* (B,E), *sty* (C,F) and *sty gram* (G-I) flowers. In A-C the flowers are shown from the top revealing the structure of petals in the second whorl, flowers in E-I are in a side view and the lower part of the wild-type flower in D has been removed to show the stamens and the carpels (front view). The genetic background in A-C and G-I is Sippe 50 and JI75 in E-F. Notice enhanced severity of the mutant phenotypes in E-F compared to B,C. The arrowhead in C points to a stamenoid petal and the arrowheads in E and F point to petaloid sepals. Whorls with homeotically altered organs are numbered in G. In H whorled organisation is disrupted and filamentous organs are indicated by an asterisk. Severe reduction in organ number is illustrated in I. Scale bars: 5 mm.

in the third whorl can be sterile or feminised. Carpels in the fourth whorl are misshapen with a broadened basal part resembling the gynoecium of *sty* and a short, sometimes narrow and split style similar to styles in *gram* flowers. *sty gram* inflorescences and flowers display several other defects such as delayed flower formation, retarded flower development and frequent abortion, resulting in irregular inflorescences (not shown). Furthermore, floral organs can be filamentous and their number reduced (Fig. 2I), in extreme cases to two sepals, two narrow radialised petaloid structures and a rudimentary gynoecium (Fig. 2F). The severity and range of defects were similar in all genetic backgrounds.

The lack of organs, stamenoidy of petals and petaloidy of sepals can be related to ectopic expansion of the C and B functions (Bowman et al., 1991; Jack et al., 1997; Krizek and Meyerowitz, 1996). Therefore, the severely enhanced *sty gram* mutant phenotype suggests that STY and GRAM co-operate to repress B and C expression outside their normal domains. In addition, the two proteins appear to be involved in the initiation and positioning of flowers and floral organs.

STY and *GRAM* co-operate in the control of vegetative development

Vegetative development of *sty gram* double mutants is severely disturbed, with irregular internode length, aberrant phyllotaxis, partial fusion of the cotyledons and arrested growth of seedlings (Fig. 3B,C). The shoot apical meristem is still functional in these seedlings, as spontaneous bursting, or manual disruption of the fused region results in organ formation and growth. None of these defects is revealed by *gram* or *sty* single mutant plants, but *sty* in the JI98 background and *gram-3* or *gram* in the JI75 background show some of the anomalies, although in a less severe form.

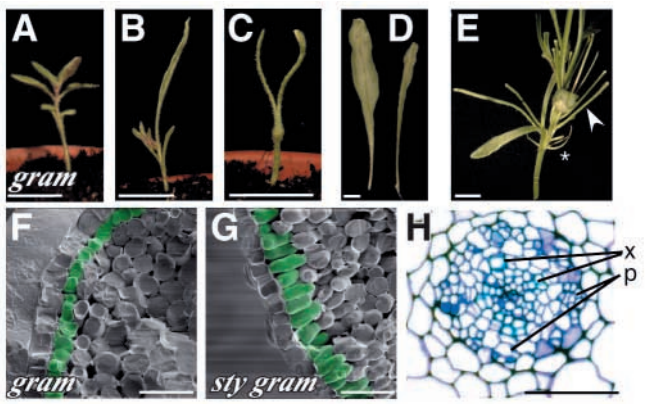


Fig. 3. Vegetative phenotypes observed with the *sty gram* double mutant. For comparison the *gram* mutant is shown in A, F and at the left in D. (A-C) 5- to 6-week-old seedlings. Notice irregular phyllotaxis in B and fused cotyledons above a 'bulge' wrapping the arrested shoot tip in C. (D) Comparison of leaves from the fourth node of 10-week-old plants. (E) Detail of a 10-week-old plant with needle-like (arrow) and filamentous leaves (*). (F,G) SE micrographs of freeze-fractured needle-like leaves. The sub-epidermal cell layer is highlighted by false green colour. (H) Histological section of the central vein in a radialised *sty gram* leaf. p and x indicate phloem and xylem elements, respectively. The mutants in E-H are in the JI75 genetic background. Scale bars: 5 mm (A-E); 50 μm (F,G) and 25 μm (H).

gram plants have narrow leaves with strips of adaxial palisade mesophyll running along the abaxial margin (Golz et al., 2004). This narrowing and partial loss of adaxial-abaxial asymmetry is not observed in *sty* mutant organs. Nevertheless, *sty gram* leaves are about 30-40% narrower than *gram* leaves (Fig. 3D). The extent of adaxialisation in the marginal regions is similar to that seen in *gram*, whereas the distance between the margin and the midrib is reduced. This reflects further reduction in cell proliferation, as the width of *sty gram* palisade leaf cells is similar to that of wild type or the single mutants. Depending on the genetic background, *sty gram* leaves more or less frequently develop as radial, filament or needle-like structures (Fig. 3E). The central vascular strand of these radial leaves has a roughly amphicribal arrangement (phloem surrounding the xylem; Fig. 3H) reflecting a loss of adaxial identity (Waites and Hudson, 1995). Surprisingly, however, the sub-epidermal tissue in these needles is more similar to adaxial palisade mesophyll than to the abaxial spongy mesophyll (Fig. 3G). This contrasts with the abaxial identity of the sub-epidermal tissue in *gram-3* needles (Golz et al., 2004) or *gram* needles that occasionally form in the JI75 background (Fig. 3F).

Taken together, these observations suggest that *STY* genetically interacts with *GRAM* for initiation and positioning of primordia and in the control of leaf polarity and growth.

Defects in *sty* mutant vascular development

The observation that *sty* influences the leaf phenotype of *gram* prompted us to study *sty* leaf morphology in detail. Measurement of the overall length and width of mature lower leaves did not reveal obvious differences compared to wild type, although segregating populations *sty* plants more often bear smaller or slightly narrower leaves at upper nodes than wild-type sisters, reminiscent of the narrow leaves of *lug* mutants (Liu and Meyerowitz, 1995; Liu et al., 2000). Interestingly, however, the venation pattern of *sty* leaves differ from wild type in that the major (primary and secondary) veins are slightly broader and the density of minor veins at the tip of the leaf is reduced (Fig. 4C,G). Furthermore, vascular strands are not properly aligned (insets in Fig. 4F-G). The severity of these defects is variable, ranging from near wild type to very aberrant. Thus, unexpectedly, *STY* has a role in vascular development.

gram leaves also show broadening of the midvein and reduction of minor venation, in particular the number of free ending veinlets (Fig. 4D,H), reminiscent of the defects seen in the *Arabidopsis fil yab3* double mutant (Siegfried et al., 1999). Venation of *sty gram* leaves is almost exclusively reduced to the extremely broad midvein and a few secondary veins, which reach or surpass the width of the wild-type midvein (Fig. 4E,I). Given that the influence of *gram* on the same process might relate to its polarity defect, the enhanced phenotype in the *sty gram* double mutant is either a synergistic or an additive effect.

STY and *GRAM* expression patterns indicate early overlap and late exclusion

The observed physical and genetic interactions between *STY* and *GRAM* suggest that these genes have overlapping expression patterns. The precise site and time of this overlap during vegetative and reproductive development was determined using both in situ mRNA hybridisation and protein

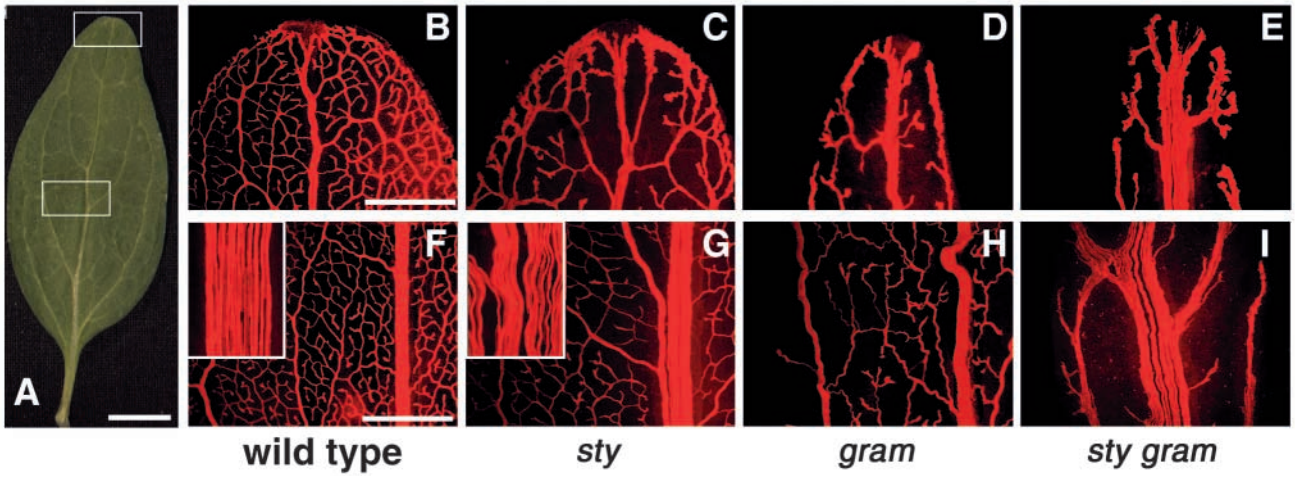
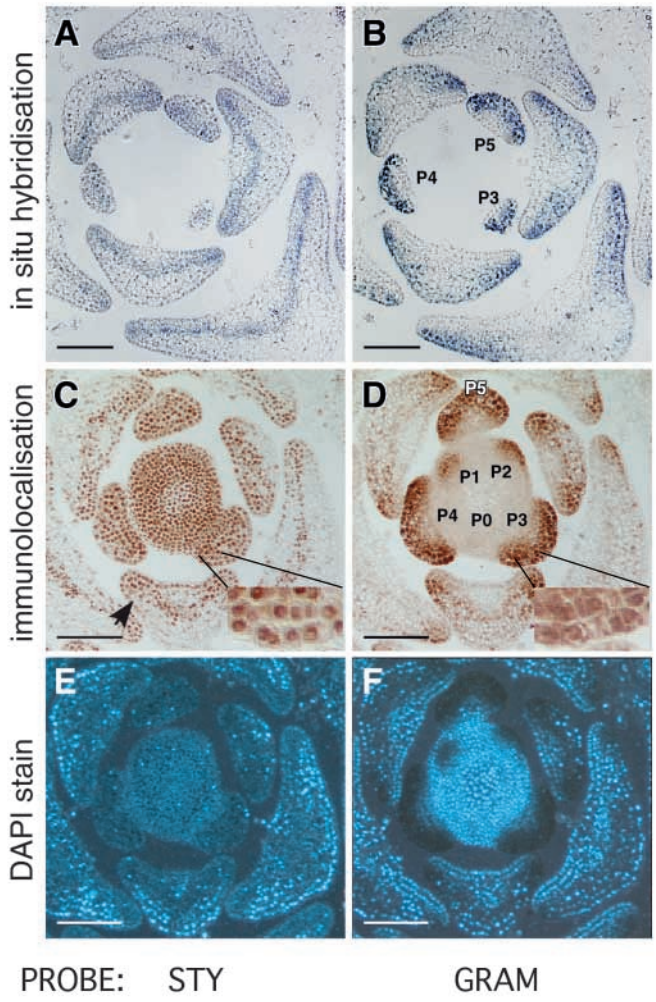


Fig. 4. Vascular skeletons revealing venation patterns in leaves. (A) A wild-type leaf with boxes indicating the approximate position in B-E (tip) and F-I (middle). The insets in F and G show additional five-fold magnifications of a midvein. Genotypes are given under the panels. Scale bars: 1 cm (A): 1 mm (B-I).

immunolocalisation. The pattern of protein and RNA accumulation is similar for each gene (Fig. 5A-D) and therefore we arbitrarily chose either RNA or protein pattern for documentation.



During vegetative development *STY* mRNA and protein is detected throughout the meristems and initiating leaf primordia (P0-P4; Fig. 5A,C). By P4-P5, expression starts to become restricted to the adaxial domain of the lamina and subsequently to the margins and (pro)-vascular tissues in expanding young leaves. During reproductive growth STY expression is abundant in the inflorescence and floral meristems as well as in the ovules (Fig. 6A,C,E,G). Initiating bracts and floral organs homogeneously express STY. Restriction of expression to vascular tissues later in development and to the adaxial regions in older organs is as observed for vegetative development. The expression pattern of *STY-L* is similar to *STY* (not shown).

In contrast to *STY*, *GRAM* mRNA and protein are excluded from the meristems and are first detected in emerging P0-P1 organs (Fig. 5D). As the organs develop *GRAM* mRNA and protein accumulate at the abaxial margins of the lamina while expression gradually decreases in the more central regions of the leaf (Fig. 5B-D). Inflorescence meristems and ovules do not express *GRAM*, but mRNA and protein are found in a broad ventral domain of initiating bracts and floral organ primordia (Fig. 6B,D,F). In older floral organs *GRAM* expression is

Fig. 5. In situ STY (left panel) and GRAM expression patterns (right panel) during wild-type vegetative development. Photographs in a row are from consecutive sections. Cross sections were prepared from the apex of 7- to 8-week-old plants. Because of floral induction the leaf primordia are arranged in a spiral order. This allows more developmental stages to be observed in one section (numbered in B and D), compared to the two to four primordia arranged in decussate phyllotaxis in apices prior to induction. (A,B) In situ mRNA expression patterns. The sections were taken from the top of a shoot and do not reveal the shoot apical meristem. (C,D) The sections for immunolocalisation show the apical meristem and very young initiating primordia. (Arrowhead in C indicates the margin region expressing STY that expands in the *gram* mutant shown in Fig. 7A) (Insets) Magnification of a small region of a P3 primordium reveals largely nuclear localisation of STY in C and additional cytoplasmic staining for GRAM in D. (E,F) DAPI counter-staining of the sections shown in C,D. Notice dark regions that do not reveal the nuclear DAPI signal due to quenching by the immunological stain. Scale bars: 100 μm.

complementary to *STY* as shown for the gynoecium in Fig. 6G,H.

In summary, potential physical interactions between GRAM and STY are limited to a short period when both genes are expressed in the same cells of initiating vegetative and floral organ primordia. Expression of both genes becomes mutually exclusive later in development.

The STY and GRAM proteins localise to the nuclei as indicated by quenching of fluorescence at sites of protein expression following a DAPI treatment (Fig. 5E-F). Intriguingly, a considerable proportion of the GRAM protein remains in the cytoplasm. This does not appear to be an artefact because cytoplasmic signals cannot be detected in the *gram-3* mutant (Fig. 7B) or in tissues where GRAM is not expressed.

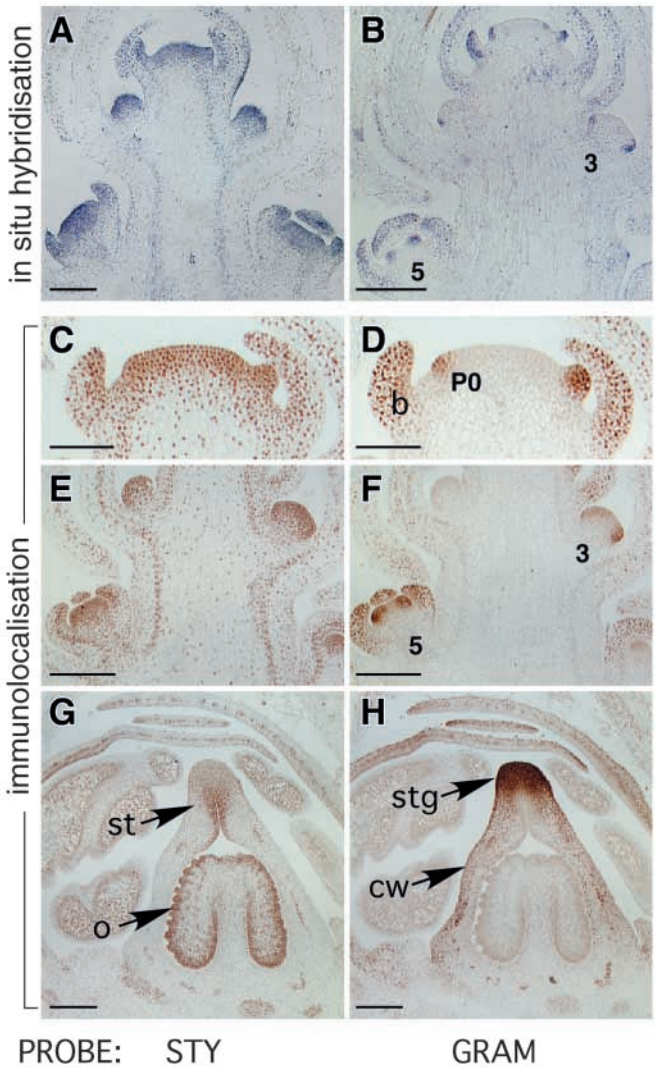


Fig. 6. In situ *STY* (A,C,E,G) and *GRAM* expression patterns (B,D,F,H) in wild-type inflorescences. The longitudinal sections are consecutive in B-F and G-H. (A,B) mRNA expression patterns. (C-H) Protein expression revealed by immunolocalisation. P0 indicates the youngest bract (b) primordium and numbers indicate floral developmental stages (Carpenter et al., 1995). The complementary pattern of STY and GRAM expression is illustrated in G,H. stg, stigma; st, style; cw, carpel wall; o, ovules. Scale bars: 200 μm (A,B,E-H); 100 μm (C,D).

The influence of leaf polarity on *STY* expression

Expression of *STY* in vascular tissues is consistent with the observed changes in vascular development in the *sty* mutant. Expression of *STY* in the adaxial domain suggests that it may establish or maintain adaxial features, a role that is not apparent from the *sty* mutant phenotype. If true, then adaxialisation of abaxial cells should result in *STY* expression. Indeed, *STY* expression expands into the abaxial margin of *gram-3* organs (arrows in Fig. 5C and Fig. 7A), a region that has adaxial identity.

Organs of *phantastica* (*phan*) mutants grown at 20-22°C show varying degrees of abaxialisation, whereas at 16-17°C organs are radially symmetric and almost completely lack adaxial identity (Waites and Hudson, 1995; Waites et al., 1998). Examining the *STY* expression pattern in *phan* mutants therefore should reveal to what extent STY is regulated by *PHAN* and/or by adaxial cell identity. Initiation of STY expression, and its early restriction to the adaxial region does not depend on *PHAN* as P4/P5 *phan* primordia express STY within their adaxial region (Fig. 7C,D). Furthermore, residual ad-abaxial asymmetry of abaxialised organs initiated at 17°C is still reflected by an adaxial STY pattern (Fig. 7C). Thus, polarised *STY* expression in *phan* mutant organs is not

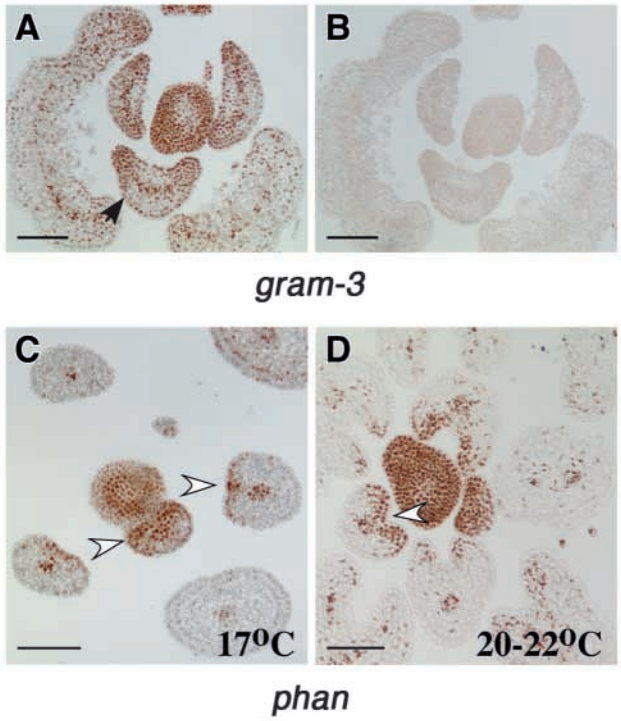


Fig. 7. STY protein expression in mutants affecting leaf abaxial-adaxial asymmetry. (A,B) Consecutive cross sections from the apex of a *gram-3* plant probed with antibody directed against STY (A) and GRAM (B). The arrowhead in A points to extended STY expression as compared to a wild-type primordium at a similar developmental stage (arrowhead in Fig. 5C). Notice lack of signal in B confirming specificity of the antibody directed against GRAM.
(C,D) Immunolocalisation of STY in cross sections of *phan* mutant apices (main shoot in C and axillary shoot in D) from plants grown at low and at intermediate temperatures. Notice adaxial localisation of the protein in partially or almost fully abaxialised leaf primordia (white arrowheads). Scale bars: 100 μm.

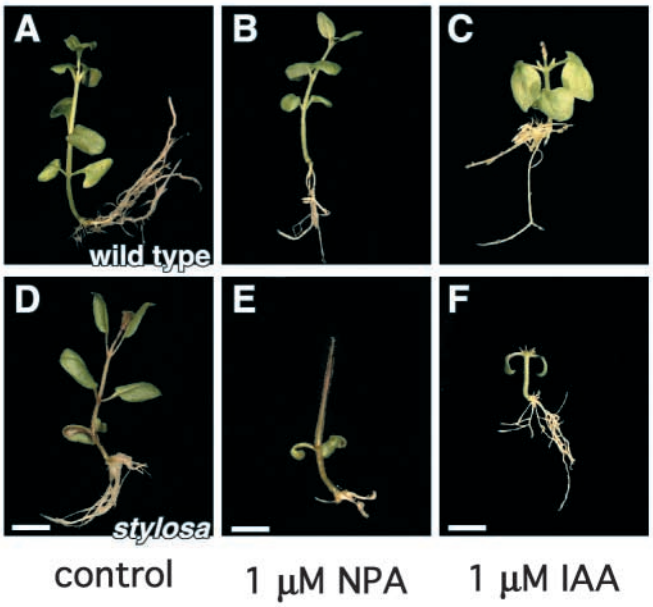


Fig. 8. Hypersensitivity of *sty* to auxins and auxin transport inhibitors. (A-C) Wild-type and (D-F) *sty* mutant seedlings after 2 weeks of growth on in vitro culture media supplemented as indicated under the panels. Scale bars: 1 cm.

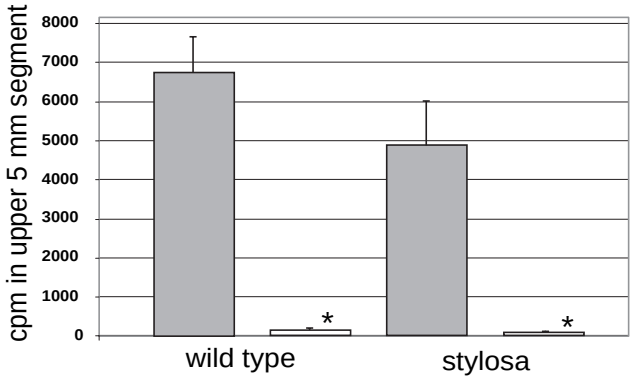


Fig. 9. Polar auxin transport in inflorescence stem sections. Average and standard deviation in a representative experiment with 20 segments are shown. The difference between wild type and *sty* mutant is significant at a confidence level >99.9%. *Physiological direction (see Materials and methods).

sufficient to promote adaxial cell identity, although *STY* expression in *gram-3* organs appropriately responds to polarity.

Hypersensitivity of *sty* mutants towards polar auxin transport inhibitors and exogenously applied auxins

Alterations in the leaf venation pattern, mild problems with phyllotaxis and the tendency of *sty* and *sty gram* mutants to fasciate (not shown) suggest that there may be local changes in auxin levels, responses or movement (Mattsson et al., 2003; Okada et al., 1991; Sieburth, 1999). To investigate this aspect of the *STY* function we applied auxin transport inhibitors and exogenous auxins to wild-type and *sty* seedlings.

Low (0.5-1 μM) concentrations of the auxin transport inhibitor NPA (or TIBA, not shown) hardly affect wild-type morphology but induce a dramatic change in *sty* seedlings (Fig. 8E). Instead of the main shoot composed of leaf-bearing internodes, *sty* seedlings develop a pin-like structure. Lateral shoots initiate from the hypocotyl after several weeks of growth and produce leaves with very broad midveins, comparable to leaves of wild-type plants grown on 10-20 times higher NPA concentration (not shown). Growth and elongation of *sty* roots are also more severely affected by NPA than those of wild-type seedlings (Fig. 8B,E). In fact, the roots of *sty* control seedlings are already shorter than wild-type ones and grow in an agravitropic manner (not shown), reminiscent of the behaviour of *Arabidopsis pin2* and *pin3* mutant seedlings (Friml et al., 2002; Müller et al., 1998). The response of *Arabidopsis lug-1* seedlings to treatment with NPA is similar to *sty* (not shown).

sty seedlings are also hypersensitive to application of auxins. Enhanced sensitivity towards IAA is revealed by epinastic leaf shape and growth defects at concentrations that do not severely affect wild-type development (Fig. 8C,F). Application of 0.5 μM 2,4D to *sty* plants results in severely retarded growth and callus formation in the hypocotyl region whereas wild-type seedlings just respond by retarded hypocotyl elongation and enhanced lateral root formation (not shown).

Hypersensitivity towards auxins and polar auxin transport inhibitors can result from reduced auxin transport. Indeed, transport measurements indicate a 20-30% inhibition of polar auxin transport in *sty* mutants compared to wild type (Fig. 9).

Discussion

STYLOSA is a GRO/TUP1-like co-repressor and interacts with YABBY proteins

The extensive similarity in morphology, genetic behaviour and hormone responses of the *sty* and *lug* mutants, together with the high degree of sequence conservation between their gene products, provides compelling evidence that *STY* is the *Antirrhinum* orthologue of *LUG*. Tight linkage between the mutant phenotype and a frameshift mutation in the *sty* mutant allele further corroborates this conclusion.

The LUG and STY proteins share similarities in domain structure with GRO/TUP1-like co-repressors (Conner and Liu, 2000). Members of the GRO/TUP1 super-family cannot directly bind DNA and are recruited to the site of their function by DNA-binding proteins, sometimes mediated by additional ‘adaptor’ proteins such as Ssn6 (Smith and Johnson, 2000). Subsequent recruitment of histone deacetylases results in transcriptional silencing (Flores-Saaib and Courey, 2000). Finding proteins interacting with STY is therefore an approach that is likely to identify proteins required for the formation of repressor complexes. Using a yeast two-hybrid screen we found that several members of the YABBY family interact with STY. Proteins in this group containing the YABBY domain (Bowman, 2000), a partial HMG-box with no DNA-binding specificity (Kanaya et al., 2002). Interestingly, interaction between GRO/TUP1-like co-repressors and HMG-domain proteins is a common feature in mammals, *Drosophila* and yeast (Brantjes et al., 2001; Cavallo et al., 1998; Deckert et al., 1995), although the HMG-domain proteins involved are quite diverse. It has been suggested that HMG proteins are architectural factors that are necessary in combination with GRO-like co-repressors and other proteins to form a

'repressosome' (see Introduction), a function that might also be associated with STY/YABBY complexes in plants. Indeed, the function or stability of the protein complex that contains STY/GRAM may well depend on additional components, which would fit with the influence of 'background factors' on the phenotype of *sty* and *gram* mutants.

By analogy to the GRO/HMG-box proteins, it is likely that the diverse functions of STY and GRAM arise from acting together as well as separately in complexes with other proteins. A further complication results from the possible partial redundancy of the *STY* function with *STY-L*, and the *GRAM* function with other members of the *YABBY* family. Despite this, developmental events in which STY and GRAM are likely to interact physically in vivo will be discussed below, along with independent STY and GRAM functions.

STY and *GRAM* in the control of floral organ identity

Several lines of evidence support the function of a STY/GRAM complex in the control of floral organ identity. Firstly, the mild floral homeotic defects in control of the outer boundary of the floral B and C functions in *sty* and *gram* mutants indicate an overlap of their function suggesting that the two genes act in the same control pathway. We assume that incomplete functional equivalence of redundant factors, or incomplete overlap of their expression patterns is responsible for the weak defects displayed by the single mutants. In support of this, *gram prol* double mutant flowers display a greater degree of homeotic conversions than *gram* single mutants (J.F.G., unpublished). Similarly, two TUP1-like proteins in fission yeast have partially redundant roles in chromatin remodelling and transcriptional repression (Hirota et al., 2003). Secondly, the combined loss of *STY* and *GRAM* results in more severe homeotic conversions than loss of either *STY* or *GRAM* alone. This is consistent with the idea that eliminating two components of a protein complex is more deleterious than eliminating just one. Third, incipient floral organs in the outer whorls concomitantly express *STY* and *GRAM* at early developmental stages, prior to establishment of the B and C functions (Bradley et al., 1993). Later this overlap resolves in a complementary pattern suggesting that the proteins perform functions other than together controlling organ identity.

Enhanced expansion of the C function to the outer whorls in *lug fil* double mutant compared to single mutant flowers has also been noted (Chen et al., 1999). The control of the C domain by the STY/GRAM and LUG/FIL complexes thus appears to be conserved between *Arabidopsis* and *Antirrhinum*, as do the respective protein interactions observed in yeast. Interestingly, several abnormal features of *Arabidopsis lug* or *fil* single mutant flowers, such as filamentous organs, reduced organ number and aberrant whorl organisation (Chen et al., 1999; Liu and Meyerowitz, 1995; Sawa et al., 1999a) are not revealed in *sty* or *gram* single mutants, but are in the *sty gram* double mutant. These differences suggest deviations in the range of control events exerted by the respective proteins or protein complexes in the two species.

Expansion of the B and C domains occurs concomitantly suggesting that the two control processes are linked. This may indicate that both the B and C control genes are regulated by STY/GRAM. Testing the physical association of STY/GRAM with regulatory regions of class B and class C genes will resolve whether this repression is direct. An alternative is that STY/GRAM govern processes preceding organ identity control, such as the timing of organ initiation or positioning of primordia, as discussed below. In accord with this idea, ectopic expansion of the C-function gene *PLENA* (*PLE*) in *sty* flowers is preceded by changes in expression of several other floral control genes suggesting that *PLE* is not the only target of *STY* regulation (Motte et al., 1998).

STY and GRAM in the control of organ initiation

Impaired initiation and positioning of leaves (aberrant phyllotaxis) and floral organs (lack of whorled organisation) is one of the severe changes during development of the *sty gram* double mutant. Since the two proteins are co-expressed in the nuclei of lateral organ primordia their interaction in the control of organ initiation is feasible. The fact that the single mutants do not display severe developmental defects in this process is perhaps due to redundancy. Positioning and emergence of lateral organs are controlled by the plant hormone auxin (Reinhardt et al., 2003). Given the observed interaction between *STY* and hormone-mediated control processes, as discussed below, it is possible that enhanced phyllotaxis defects in the *sty gram* mutant are related to impaired auxin perception or movement.

STY in the control of leaf polarity

The *sty* mutant does not display obvious loss of organ polarity, perhaps because of redundancy with the *STY-L* gene. However, two observations suggest a redundant role for *STY* in the control of adaxial fate. Firstly, *STY* expression becomes adaxially restricted similar to *Arabidopsis* genes such as *PHABULOSA* (*PHB*) that promote adaxial identity in lateral organs (McConnell et al., 2001). This restriction of *STY* occurs in P4 primordia, subsequent to adaxial restriction of *AmPHB* during late stage P1 (Golz et al., 2004) and thus *STY* is not likely to be involved in the initial establishment of adaxial asymmetry. Consistent with this, abaxialised *phan* mutant primordia retain spatially correct adaxial *STY* expression, suggesting that asymmetric *STY* expression is independent of *PHAN*. Nevertheless, *STY* expression expands into the adaxialised margins of *gram-3* organs, indicating that *STY* expression can follow adaxial fate. Secondly, the radialised needle-like leaves that develop in the *gram sty* double mutant suggest a common role of *GRAM* and *STY* in leaf asymmetry, supported by co-expression of the two genes. However, *gram sty* needles, unlike the abaxialised needles occasionally forming in *gram* mutants, show both abaxialised and adaxialised characters. The reason for this is presently not clear and the role of *STY* in the control of adaxial identity remains enigmatic.

Independent roles of STY and GRAM in the control of leaf lamina growth

Clonal analysis suggests that *GRAM* promotes cell divisions in marginal cells of leaf primordia. In the *gram* mutant growth at the margins is reduced, but the effect on leaf width is in part compensated by enhanced cell divisions in more central regions (Golz et al., 2004). Reduction of *sty gram* leaf width indicates that *STY* is needed for compensatory growth and therefore that *STY* might control proliferation in the central regions of the wild-type leaf. In accordance with this, *STY* is expressed during the phase of expansion in the vascular tissue

and at the junction between abaxial and adaxial regions of young leaves. The role of *STY* in promoting growth weakly manifests in reduced leaf width in the *sty* mutant, perhaps because enhanced cell divisions in the margin replace cells derived from more central regions. Reduced width of *sty gram* leaves might thus reflect independent roles of *GRAM* and *STY* in the control of lateral growth.

STYLOSA in the control of venation and hormone-mediated processes

A function of *STY* during vascular development is underscored by prominent expression of the gene in provascular and vascular cells, and is confirmed by impaired venation and changes in auxin responses/polar auxin transport in the *sty* mutant. In contrast, *GRAM* is not expressed in provascular cells and *gram* mutants just weakly respond when grown on auxins or auxin transport inhibitors (N.E., unpublished). Aberrant venation in *gram* might be the consequence of polarity and growth defects, as these features are tightly linked (Dengler and Kang, 2001; Waites and Hudson, 1995). Thus, the enhancement of vascular defects in *sty gram* results from the combined effects of both genes.

Our observations on impaired development of major and minor leaf veins and enhanced responses of the *sty* mutant to auxins or polar auxin transport inhibitors strongly suggest a role of *STY* in producing or mediating hormone-dependent signals. The precise site at which *STY* is involved in auxin signalling or transport is not clear. Transport measurements suggest partial inhibition of polar auxin transport, but give no hint whether this is due to a direct effect of STY on transport or an indirect effect of other *STY*-controlled events. Since most hormone-related processes are interconnected (Coenen and Lomax, 1997; Ephritikhine et al., 1999) it is possible that other hormones are primarily influenced by *STY*.

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