The **Handlebars** gene is required with **Phantastica** for dorsoventral asymmetry of organs and for stem cell activity in *Antirrhinum*

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**SUMMARY**

In angiosperms, individual lateral organs and whole flowers may develop asymmetrically along their dorsoventral axes. Dorsoventral asymmetry of *Antirrhinum* leaves requires activity of the **Phantastica** gene and other factors acting redundantly with it. We describe the effects of a mutation in the **Handlebars** gene, identified as an enhancer of the **Phantastica** mutant phenotype. Genetic analysis suggests that **Handlebars** functions redundantly with **Phantastica** to promote dorsal fate in lateral organs and to maintain activity of stem cells within shoot apical meristems. **Handlebars** appears dispensable in vegetative development but is needed for asymmetry of petals along the dorsoventral axis of the flower as a whole. This suggests that common mechanisms may control dorsoventral asymmetry in lateral organ primordia and in floral meristems.

Key words: *Antirrhinum majus*, dorsoventrality, shoot apical meristem, **Phantastica**, **Handlebars**

**INTRODUCTION**

The shoots of higher plants show obvious apical-basal (AB) polarity. Each is formed by a shoot apical meristem (SAM) at its tip and therefore grows and matures apically. The organs and secondary meristems produced on the flanks of the SAM elaborate further asymmetry in response to AB polarity. In *Antirrhinum*, for example, leaves are flattened in a plane perpendicular to the AB axis of the vegetative stem and petals have different identities according to their positions along the dorsoventral axis of the flower, coincident with the AB axis of the inflorescence stem (Carpenter and Coen, 1990; Waites and Hudson, 1995).

Specification of dorsoventral (DV) asymmetry in lateral organs and flowers appears closely coupled to SAM function. Lateral organ primordia and floral meristems show dorsally or ventrally restricted gene expression at, or before, initiation suggesting that their DV asymmetry is specified within the SAM (Coen et al., 1990; Long and Barton, 2000; Luo et al., 1996; Luo et al., 1999; Lynn et al., 1999; Siegfried et al., 1999). Loss-of-function mutations in genes needed for dorsal identity leads to development of ventralised, radially symmetrical organs or flowers suggesting that loss of dorsal fate allows ventral fate to occur by default (Almeida et al., 1997; Bohnert et al., 1998; Lynn et al., 1999; Moussain et al., 1998; Waites and Hudson, 1995). Surgical experiments have further suggested that dorsal identity in lateral organs is dependent on signals originating apically within the SAM (Sussex, 1951), consistent with the observations that organs or flowers produced ectopically from the apex of the SAM, rather than its flanks, lack dorsal identity and DV asymmetry (Bradley et al., 1996a; Lynn et al., 1999), and that mutations causing loss of SAM activity may also lead to production of ventralised organs (Pickett et al., 1996). Such signals would provide a mechanism by which AB polarity of the SAM could be translated into DV asymmetry of organs and flowers. Not only is DV asymmetry of organs dependent on SAM function, but activity of the SAM also appears dependent on signals from lateral organs (Waites et al., 1998), and dorsal cell identity in lateral organs is both necessary and sufficient for activity of secondary SAMs formed in their axils (McConnell and Barton, 1998).

We previously identified a requirement for the *myb*-like **Phantastica** (**Phan**) gene in asymmetry of lateral organs in *Antirrhinum* (Waites et al., 1998). Although **Phan** mutants fail to initiate organs and show quiescence of the SAM at restrictive low temperatures, they produce ventralised leaves and petals at semi-permissive temperature, indicating that **Phan** promotes dorsal organ fate. However, **Phan** is not absolutely necessary for dorsal fate, because even null **Phan** mutants develop normally at permissive temperatures, implying that it acts redundantly with factors that are sensitive to cold.

To identify such factors, we have carried out a screen for novel mutations that enhance the **Phan** mutant phenotype and discovered the recessive **handlebars** (**hb**) mutation. Genetic analysis suggests that **Hb** and **Phan** are required redundantly for dorsal cell fate in organs and to promote stem cell activity in meristems, and the phenotypes of **phan hb** double mutant reveal additional functions of **Phan**. Although **Hb** is dispensable in vegetative development, it is needed for asymmetry of petals along the dorsoventral axis of the
flower, suggesting that mechanisms controlling dorsoventral asymmetry are conserved in flowers and lateral organs.

MATERIALS AND METHODS

Plant genetics

Genetic screens were carried out on phan-607, phan-552 phan-250 or phan-249 homozygotes (Waites and Hudson, 1995), into which active transposons had been introduced by crossing to the wild-type line JI.98, or its derivative, JI.605 (Hudson et al., 1993). Homozygous phan mutants were self-pollinated to produce M₀ plants which were shifted to 17°C before flowering to increase transposition, and self-pollinated to produce approx. 600 M₁ progeny. Three flowers from each M₁ plant were self-pollinated and an M₂ family of approx. 200 seedlings was grown from each fruit, giving a total M₂ population of about 120,000 plants. M₂ seedlings with novel phenotypes were grown to maturity with several of their M₁ siblings.

The phan mutation was germinally unstable reverting to wild type in approx. 5% of gametes, suggesting that was caused by an active transposon. To test whether enhancement of the phan mutant phenotype and effects on petal development were attributable to the same mutation, different phan/+ plants were self-pollinated and 12 wild-type progeny (i.e. those having inherited a revertant Phan+ allele and a Phan+ allele) self-pollinated. All families derived from Phan+/phan parents segregated wild-type, phan, and Phan+ double mutant phenotypes in ratios approximating to 9:3:1, indicating that all independent reversions of the phan petal phenotype were accompanied by loss of the ability to enhance the phan mutant phenotype. Therefore Phan+, rather than a closely linked mutation, appeared responsible for the effects on petal development and the phan mutant phenotype.

Plants mutant for either Phan or Phan and one of the floral asymmetry genes Cyclidea, Dichotoma, Radialis or Divaricata, were obtained by crossing to Phan or phan single mutant homozygotes. Double mutants were identified in the F₂ progeny on the basis of phenotypic frequency and their genotypes confirmed by back-crossing to the relevant single mutant parents.

The phan-164 allele was obtained in the mutant line indisposita (MAM164) from IPK-Gatersleben, as were lines homozygous for the floral asymmetry mutants described previously (Almeida et al., 1997; Carpenter and Coen, 1990; Luo et al., 1996; Luo et al., 1999).

Molecular biology

Southern hybridisation of DNA from phan-164 homozygotes revealed an insertion of at least 4.5 kb between a PsI site within the Phan coding region and a BglII site in the 3’UTR (nucleotides 995-1388 of the full-length cDNA sequence, A3005886). PCR, using a Phan-specific primer spanning the PsI site and primers complementary to different Antirrhinum transposons followed by DNA sequencing, identified the insertion as a copy of the Tam7 transposon.

To analyse expression of Phan mRNA, 1 μg of total RNA was obtained from vegetative shoot apices (leaves <3 mm in length) and used in cDNA synthesis with the oligo(dT)-anchor primer, Qo (Frohman, 1995). Relative abundance of Phan CDNA obtained from the equivalent of 10 ng of total RNA was then estimated by 20 cycles of PCR with a Phan-specific primer spanning the PsI site and the anchor primer Qo (essentially 3’-RACE) followed by Southern hybridisation. Use of two Phan-specific primers capable of amplifying the first 506 bp of the coding region (which is uninterrupted in all mutant alleles) gave equivalent results. Comparable efficiency of cDNA synthesis was confirmed by amplification with two primers specific for the Antirrhinum Polyubiquitin gene (X67957), kindly provided by Dr Maria Perez, John Innes Centre. To determine the sequences of transcripts produced from the phan-249 and phan-164 alleles, the products of first-round 3’-RACE reactions were further amplified with nested primers (Phan-specific and Q) and sequenced.

Microscopy and imaging

Optical sections of Antirrhinum seedlings were made by confocal laser scanning microscopy as described for Arabidopsis (Clark et al., 1993). Histological sections and scanning electron microscopy used techniques described previously (Waites and Hudson, 1995). In situ hybridisation to detect Amsm mRNA was performed as described previously (Waites et al., 1998). Living petals and flowers were imaged directly on a flatbed scanner.

RESULTS

Transposon mutagenesis identifies handlebars as an enhancer of the phantastica mutant phenotype

To identify modifiers of the phan mutant phenotype, mutagenic transposons were introduced into lines carrying different phan mutant alleles, and about 120,000 M₂ seedlings screened for more severe phenotypes at the semi-permissive temperature of 20°C. The strongest mutant phenotype was identified in three families of a family homozygous for the mis-sense mutation, phan-249. It was called handlebars (hb) because of the shape of the mutant seedlings (Fig. 1A).

Because the new mutants were sterile, their phan single mutant siblings were self-pollinated. Several gave rise to progeny with phan and hb mutant phenotypes in a ratio of approximately 3:1, suggesting that the hb mutant phenotype was caused by a single recessive mutation, hb. To introduce the hb mutation into a Phan+ background, phan/phan hb/+ plants were crossed to their wild-type progenitor. Assuming that the two loci were unlinked, half resulting F₂ families (those that had inherited the hb mutant allele) were expected to consist of wild-type plants, phan single mutants, hb single mutants and phan hb double mutants in a ratio of 9:3:3:1. However, wild type, phan single and phan hb double mutant seedlings were observed in ratios approaching to 12:3:1. Failure to identify hb single mutant seedlings and the unexpectedly high frequency of wild-type seedlings suggested that the hb mutation had no effect on vegetative development in a Phan+ background. Independent segregation of phan and hb alleles in these, and subsequent, experiments also indicated that the two loci were unlinked.

Phan and Hb are required for formation of the embryonic shoot apical meristem

At permissive temperatures of 17°C and above, all phan mutant embryos developed a functional shoot apical meristem (SAM; Fig. 1A). In contrast, 98% of their phan hb double mutant siblings lacked a functional SAM at either permissive or restrictive temperatures and their cotyledons met at the shoot apex. The remainder (2%) produced a single leaf apically between the cotyledons, suggesting the presence of embryonic SAM cells that were consumed in organ formation (right-most seedling in Fig. 1A).

Scanning laser confocal microscopy of emerging seedlings revealed no difference between the SAM structure of wild type and phan single mutants (distinguished by the phenotypes of their expanding cotyledons). Both developed a central zone (CZ) of about 50 cells flanked by more strongly staining cells of the peripheral zone (PZ) from which leaf primordia would
subsequently initiate (Fig. 1B). In contrast, the CZ appeared to be absent in phan hb double mutants, replaced by a group of strongly staining PZ-like cells that lacked the layered arrangement of wild type. In about 10% of seedlings, these cells subsequently divided and came to resemble cells of organs (Fig. 1B). These observations suggested that Phan and Hb are together required for formation or maintenance of the CZ, but not the PZ. To further investigate cell identity, in situ hybridisation was carried out to detect expression of Amstm, an Arabidopsis homologue of the SHOOT MERISTEMLESS (STM) gene of Arabidopsis, which is expressed in SAM cells, but not in lateral organs or their initials (waites et al., 1998). Amstm expression in hb single mutant seedlings was indistinguishable from wild-type expression (Fig. 2A). However, low levels of expression were also observed in cells between the cotyledons of phan hb double mutant seedlings (Fig. 2B), suggesting that these cells retained aspects of SAM identity.

**Phan and Hb maintain postembryonic meristems**

After several weeks, all phan hb double mutants developed ectopic, adventitious SAMs from either the basal hypocotyl (approx. 70%; Fig. 3B) or both the hypocotyl and needle-like cotyledons near their junctions with cotyledon blade tissue (approx. 30%; Fig. 3C). Initiation of postembryonic meristems from hypocotyl tissue also follows surgical ablation of the SAM in wild-type seedlings (Fig. 3D,E).

The ectopic SAMs had a wild-type arrangement of three cell layers but were smaller and initiated more closely spaced organ primordia (Fig. 3F). They remained consistently smaller than wild type or phan single mutant SAMs during postembryonic growth (Fig. 3G-I), gave rise to thinner stems (Fig. 3M) and terminated frequently in production of ectopic, apical leaves (Fig. 3J). Because termination of shoots and apical organ formation is a characteristic of Arabidopsis plants carrying weak *stm* mutations (endrizzi et al., 1996) we examined the effects of phan and hb mutations on expression of the *Antirrhinum* homologue, *Amstm*. Expression was detected in SAM cells of wild-type vegetative apices and in cells at leaf axils from which axillary SAMs would subsequently form (Fig. 2C). It was unchanged in hb single mutants (data not shown) and appeared similar in phan single mutant apices and in active *hb* phan double mutant apices (Fig. 2D,E). It was also consistently detectable in an internal domain of *phan* apices that had ceased production of terminal organs (Fig. 2F). This suggested that loss of *Amstm* expression was not responsible for loss of SAM activity in *phan* hb mutants.

**phan hb** double mutants showed a decreased frequency of lateral branching that correlated with reduced axillary SAM formation. Whereas all wild-type leaf axils formed SAMs, only approx. 50% of phan hb leaves showed any evidence of axillary meristems, even in shoots in which the primary SAM had previously terminated in organ formation (Fig. 3M). Loss of axillary SAMs is also a characteristic of phan single mutants where it occurs at a lower frequency and only in the axils of needle-like, ventralised leaves (approx. 30% for such leaves in the phan-250 mutant). In both phan single and phan hb double mutants, the SAMs formed in the axils of needle-like leaves developed later and more apically on the stem (Fig. 3L), as normally seen in wild type for secondary axillary SAMs formed at the junction of a lateral shoot and the primary stem. This suggested that Hb enhances the effect of phan mutations on axillary SAM formation and, because loss or retardation of axillary meristem formation is associated only with ventralised leaves, that it may be a consequence of loss of dorsal identity in the adjacent organ. All axils of *phan* and *phan hb* double mutants showed expression of *Amstm* RNA (Fig. 2C-F), suggesting that loss of axillary SAM activity did not result from reduced *Amstm* expression.

**phan hb** double mutants could make the transition to reproductive growth, as evidenced by the increased density of hairs produced on the stem and bract-like organs (Bradley et al., 1996b). However, the transition was delayed relative to wild-type plants which produced 20–30 leaves before flowering, but similar to *phan* mutants which produced up to 40 leaves under the same conditions. In addition to lacking axillary vegetative SAMs, phan hb double mutants produced flowers only rarely in the axils of bracts (<2%). Each flower typically consisted of a whorl of reduced sepals surrounding a collection of rudimentary green organs of uncertain identity (Fig. 3N).

**Hb and Phan are needed for dorsoventral asymmetry of cotyledons, leaves and bracts**

The cotyledons of phan single mutants are broader than wild type (Fig. 1A). In contrast, the cotyledons of most phan hb double mutant seedlings (86%) consisted of needle-like, ventralised tissue for most of their length with a reduced blade distally (Fig. 1A). The remaining 14% showed a weaker phenotype with less of the cotyledon developing ventralised morphology. In both cases, the phan hb double mutant cotyledons showed a more severe loss of dorsoventral asymmetry than phan single mutants (which never produce needle-like cotyledons), suggesting that *Phan* and *Hb* act redundantly in the embryo to specify dorsal cotyledon fate.
At all temperatures, the adventitious meristems of \textit{phan hb} double mutants formed shoots with needle-like, ventralised leaves similar to those of \textit{phan} single mutants grown at low temperatures (Fig. 4A), suggesting that \textit{Hb} also shared a role with \textit{Phan} in promoting dorsal leaf fate. Less than 2\% of leaves produced a small area of dorsalised blade tissue even at the permissive temperature of 25$^{\circ}$C, at which all \textit{phan} mutant leaves resemble wild type. Histologically, the leaves of \textit{phan hb} mutants resembled the needle-like leaves of \textit{phan} single mutants, consisting of the tissues associated with the ventral midrib of wild-type leaves in a radially symmetric arrangement (Fig. 4B). In this respect, they differed from the petioles of wild-type leaves (Fig. 4B) which were narrower than the lamina but showed dorsoventral asymmetry.

The \textit{hb} mutation enhances all \textit{phan} mutant phenotypes

The \textit{hb} mutation strongly enhanced the phenotype of the \textit{phan}-249 mutant. Because \textit{phan}-249 conditioned an intermediate phenotype (Fig. 5A) it was likely to encode reduced Phan activity. Therefore its interaction with the \textit{hb} mutation did not suggest whether \textit{Hb} and \textit{Phan} normally function independently or sequentially. To distinguish between these possibilities, the structure and expression of an allelic series of \textit{phan} mutations was analysed and their effects in \textit{Hb}+ and \textit{hb} genetic backgrounds compared.

The \textit{phan}-250 mutation conditions a strong mutant phenotype and is caused by an insertion within the 5$^{\prime}$UTR that reduces abundance of \textit{Phan} transcripts to levels undetectable by in situ hybridisation. The intermediate \textit{phan}-249 allele carries an insertion of the Tam2 transposon towards the 3$^{\prime}$ end of the \textit{Phan} coding region (Fig. 5B; Waites et al., 1998).

A very weak \textit{phan}-like phenotype was observed in the flowers of the classical mutant \textit{indisposita} (Fig. 5A) and shown to be caused by a novel mutant allele, named \textit{phan}-164. Disruptions to dorsoventrality of \textit{phan}-164 mutant leaves was observed only at temperatures below 20$^{\circ}$C, and \textit{phan}-164 mutant petals were only slightly reduced (Fig. 5A). Southern hybridisation suggested that \textit{phan}-164 carried an insertion and sequence analysis revealed this to be a copy of the Tam7 transposon which had inserted within the \textit{Phan} coding region,

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**Fig. 2.** Expression of the Amstm gene. In situ hybridisation was used to detect expression of the Amstm gene in (A) wild-type seedlings, (B) \textit{phan hb} double mutant seedlings, (C) wild-type vegetative apices, (D) vegetative apices of \textit{phan} single mutants, and \textit{phan hb} double mutant apices that were still active (E) or had terminated growth (F). Arrowheads indicate expression in the axils of leaves.

**Fig. 3.** Effects of \textit{phan} and \textit{hb} mutations on apical meristems. (A) Wild-type seedling with a functional SAM (arrow) and \textit{phan hb} double mutant seedlings with adventitious SAMs derived from (B) the hypocotyl or (C) needle-like cotyledons and hypocotyl. (D,E) Wild-type seedlings produce similar adventitious SAMs following ablation of the original SAM. (F) Scanning laser confocal micrographs of a \textit{phan hb} double mutant seedling with an adventitious SAM derived from the cotyledon (c; upper inset) and a non-functional SAM at the hypocotyl apex (h; lower inset). (G-J) Comparison of SAMs from (G) wild type, (H) \textit{phan} single mutant, (I) \textit{phan hb} double mutants in which the SAM is still active, or (J) has recently terminated in leaf formation. (K-M) The leaf axils of (K) wild type, (L) a \textit{phan} single mutant and (M) \textit{phan hb} double mutant pet, leaf petiole; ax, axillary meristem. (N) A flower of the \textit{phan hb} double mutant consisting of sepals (sep) surrounding organs of uncertain identity.
45 bp downstream from the insertion site of the Tam2 transposon in \textit{phan}-249 (Fig. 5B).

The relative levels of transcripts produced from \textit{phan}-249, \textit{phan}-250, \textit{phan}-164 and \textit{Phan}+ alleles were analysed by reverse transcription of RNA from shoot apices followed by amplification (RT-PCR) with either \textit{Phan}-specific primers flanking a transcribed region, which is uninterrupted in all alleles, or one \textit{Phan}-specific primer and one primer complementary to the poly(A) tail (3' RACE). Transcripts were undetectable in \textit{phan}-250, consistent with previous in situ hybridisation data, and present at intermediate levels in \textit{phan}-164 (Fig. 5D). Although \textit{phan}-249 conditioned a more severe mutant phenotype than \textit{phan}-164, its transcripts were consistently detected at higher, near wild-type, levels suggesting that its protein product was less abundant or less active than that of \textit{phan}-249. To investigate protein-coding capacity further, 3' RACE products from \textit{phan}-164 and \textit{phan}-249 were sequenced. Both produced transcripts that terminated at one of several polyadenylation sites within their transposon insertions, and had the potential to encode novel proteins in which C-terminal regions of Phan were replaced by novel amino acids encoded by the transposons (Fig. 5D).

Although the phenotype conditioned by the \textit{phan}-164 mutation was considerably less severe than that of the null mutation, \textit{phan}-250 in a \textit{Hb}+ background, their effects were equivalent in a \textit{hb} mutant background and indistinguishable from those described for \textit{phan}-249 (data not shown).

**A requirement for \textit{Hb} in floral asymmetry**

The wild-type flower shows distinct asymmetry along the dorsoventral axis of the flower which coincides with the apical-basal axis of the inflorescence. The five petals show three different identities – dorsal, lateral and ventral – according to their position along this axis (Fig. 6A). One aspect of petal identity results from differential growth: dorsal petals are broader and longer than the ventral petal, with lateral petals intermediate in size. In addition, each petal shows internal asymmetry along the same axis. Thus the two halves of each dorsal petal lobe differ in pigmentation and shape and their upper edges are united for most of their length, while their lower edges are free (Fig. 6A). Similarly, the two lateral petals show asymmetry in shape and coloration with intensely yellow pigmented cells confined to their lower-most parts. The ventral petal (which straddles the dorsoventral axis of the flower) also shows less intense pigmentation in its upper parts (towards its edges) and its lower part (towards its centre).

All five petal lobes of \textit{hb} single mutants showed reduced internal asymmetry and were narrower than wild type. Dorsal petal lobes were more symmetrical in shape and pigmentation...
Fig. 6. *Hb* is required for floral asymmetry. (A) The wild-type corolla contains two dorsal petal lobes (d), two lateral lobes (l) and one ventral lobe (v) shown here detached from the corolla tube with their dorsal surfaces uppermost. Each type of petal lobe has a characteristically asymmetric shape and distribution of pigment. The corresponding petal lobes of *hb* single mutants show reduced asymmetry in both shape (stars) and pigmentation (arrows). (B) In the wild-type corolla tube (viewed from below), the ventral petal is characterised by a bulge in its proximal region and two stripes of pale pigmentation mark its boundaries with lateral petal. In *hb* single mutants, the bulge extends laterally. Reduced activity of *Cyc* or *Rad* genes has a similar effect (data not shown).

and both their upper and lower edges were free (Fig. 6A), suggesting that *Hb* is involved in specifying the fate of the upper region in petal lobes. Lateral petal lobes of *hb* mutants were also more symmetrical in shape and the intensely pigmented cells characteristic of lower regions were also present in their upper parts, suggesting a similar role for *Hb* in lateral petal lobes. In addition, the ventral petal lobe of *hb* mutants was reduced in size and showed more symmetrical pigmentation. The *hb* mutation also affected dorsoventral asymmetry of the petal tube. In wild type, the ventral petal tube was marked by two stripes of palely pigmented cells at its upper edges and forms a basal bulge from its lowest part. The width of the ventral petal tube was unaffected by the *hb* mutation (Fig. 6B). However, its upper parts also contributed to formation of a bulge which was therefore larger than in wild type. This suggested that *Hb* is also needed to specify upper fate in the ventral petal tube.

In contrast to the *hb* mutation, *phan* mutations reduce petal size to a degree characteristic of each allele (Fig. 4A; Waite and Hudson, 1995), but do not affect internal asymmetry of petal lobes (Fig. 7C) except when lobes are reduced to completely symmetrical needles. This suggested that *Hb* might regulate a different aspect of petal development to *Phan*.

Internal asymmetry of upper petals also requires activity of the *Dichotoma* (*Dich*) gene (Luo et al., 1999). The dorsal petal lobes of *dich* mutants resemble those of *hb* single mutants in that their upper edges are free and their shape is more symmetrical than wild type (Fig. 7E). However, *dich* mutations, unlike *hb*, affect only dorsal petal lobes and do not noticeably alter their width. *hb dich* double mutants produced dorsal petal lobes that were more symmetrical than in either single mutant (Fig. 7E), suggesting that the two genes function independently in different processes. The dorsal petals were also narrower than wild type, as in *hb* single mutants, and lateral and ventral petals affected in the same way as in *hb* single mutants.

In addition to *Dich*, which is required only for internal asymmetry of dorsal petals, three other genes act in combination to specify petal identity along the dorsoventral floral axis and influence the internal asymmetry of individual petals. Loss of the dorsalising effect of either *Cycloidea* (*Cyc*) or *Radialis* (*Rad*) causes petals in lateral positions to resemble ventral petals and the lower part of each dorsal petal to assume lateral identity (Fig. 7D,F). In contrast, *Divaricata* (*Div*) is required for ventral petal identity and acts dose-dependently, in combination with *Cyc*, to specify asymmetry of lateral petals (Fig. 7G).

The effects of *hb* on petal width and asymmetry were unaffected by reduced activity of *Cyc*, *Rad* or *Div* (Fig. 7D,F,G). This suggested that *Hb* acts on petal asymmetry independently of these genes. Similarly additive phenotypes were observed in flowers of plants mutant for both *Phan* and each of the floral asymmetry genes (data not shown).

**DISCUSSION**

**Redundancy of Phan and Handlebars**

The *Phantastica* (*Phan*) gene is needed for dorsal identity in leaves and petal lobes of *Antirrhinum*, for initiation of lateral organs and to maintain activity of shoot apical meristems (SAMs) non-cell autonomously. However, *Phan* is dispensable at higher temperatures, suggesting that it is redundant with respect to a parallel cold-sensitive pathway (CSP; Waite et al., 1998). The CSP must itself be redundant, because wild-type plants develop normally at low temperatures.

The ability of the recessive *handlebars* (*hb*) mutation to enhance both null and weak *phan* mutant phenotypes suggests that *Hb*, like the CSP, overlaps in function with *Phan* but is expressed independently. Three additional observations are further consistent with *Hb* acting as a component of the CSP: (i) *Hb*, like the CSP, appears to be redundant, (ii) reduced activity of either the CSP or *Hb* confers a similar phenotype in a *phan* mutant background, as expected of factors acting in parallel with *Phan*, and (iii) the *hb* single mutant phenotype is insensitive to temperature, suggesting that *Hb* does not act in parallel to the CSP. However, the effects of cold and the *hb* mutation on the *phan* mutant phenotype are not entirely equivalent: *phan* single mutants at restrictive temperatures fail to initiate organs whereas organ initiation is unaffected in *phan hb* double mutants. This weaker effect of the *hb* mutation, compared to reduced activity of the CSP, can be most easily explained if *Hb* functions in a branch of the CSP which comes after its cold-sensitive step and is not involved in organ initiation. Alternatively, the *hb* mutation might be weak and allow sufficient CSP activity for organ initiation but not for organ dorsoventrality or meristem activity.

One explanation for the redundancy of *Phan* is that a related gene has similar functions. However, although the *Antirrhinum* genome contains one additional *Phan*-like gene, this does not map to *Hb* (R. W., unpublished).
The roles of Phan and Hb in the shoot apical meristem

Phan acts redundantly with the CSP to maintain activity of all shoot apical meristems (SAMs) because all SAMs of phan mutants cease growth and organ initiation at low temperatures but are capable of reinitiating growth on return to permissive temperatures (Waite et al., 1998). Although reduced activity of Hb alone had no effect on SAM function, phan hb double mutant embryos either lacked a SAM or a recognisable central zone (CZ) and terminated in organ formation, suggesting that Phan and Hb together promote formation or maintenance of the CZ. Because Phan expression is restricted to organs forming outside the CZ, this must involve cell-cell signalling. Hb could therefore be required in organs to generate the signal or elsewhere in the SAM to transmit or respond to it. The requirement for Phan and Hb in SAMs is not absolute because all phan hb mutant seedlings retain the ability of wild-type plants to initiate adventitious SAMs which also show defects that are consistent with impaired CZ function: they remain smaller than wild type, produce narrower shoots and frequently appear to terminate in production of ectopic organs. The difference between the loss of the CZ to ectopic organs in phan hb double mutants and the quiescence of the SAM in phan mutants at restrictive temperatures can be explained by the organ promoting activity proposed to remain in phan hb mutants.

In Arabidopsis, CZ function is influenced by at least two genetic pathways. Activity of three CLAVATA (CLV) genes restricts the size of the CZ, partly by limiting expression of the meristem-promoting WUSCHEL (WUS) gene (Brand et al., 2000; Jeong et al., 1999; Schoof et al., 2000). Therefore the quiescence or temporary loss of organisation seen in SAMs of wus mutants can be interpreted as the opposite effect to clv mutations—that is loss of CZ activity (Mayer et al., 1998). This phenotype resembles that of phan mutant SAMs at restrictive temperatures, consistent with Phan and Hb promoting CZ activity via a WUS-like factor. In contrast, mutations in other Arabidopsis genes, including SHOOT MERISTEMLESS (STM), cause loss of the CZ and production of ectopic organs (Endrizzi et al., 1996), as observed in phan hb double mutants, suggesting that Phan and Hb are required for an STM-like activity. However, transcripts from the Antirrhinum STM homologue, Amstm, were detectable in phan hb apices, even after they had ceased activity, suggesting that the requirement for Phan and Hb in the SAM does not reflect a role in promoting Amstm transcription. Similarly, loss of SAM activity in Arabidopsis stm mutants requires the activity of the Phan orthologue. ASYMMETRIC LEAVES1 (ASI) because removal of both AS1 and STM activity allows SAMs to grow normally (Byrne et al., 2000), suggesting that STM might promote CZ activity only by repressing organ fate. In contrast, the role of Phan appears independent of organ fate repression, because at low temperature phan mutants lack both organs and an active SAM. This suggests that Phan is unlikely to act solely on an STM-like factor. A further possibility is that it promotes expression of a gene similar in function to PINHEAD/ZWILLE (ZLL) of Arabidopsis which is expressed at low levels in a domain comprising the SAM and dorsal organ initials (Lynn et al., 1999). zll mutant embryos resemble phan hb double mutants, in forming a SAM-like structure that terminates in organ formation (McConnell and Barton, 1995; Moussain et al., 1998). The related ARGONAUTE (AGO) gene is expressed ubiquitously (Bohmert et al., 1998), but is required for dorsal organ fate (as are Phan and Hb), probably because it shares dorsalising function with ZLL (Lynn et al., 1999). AGO and ZLL are required to maintain STM activity although they appear to have other roles in the meristem, as proposed for Phan and Hb. These phenotypic similarities suggest that Hb might be required for a function similar to that of ZLL and AGO.

Defects in SAM formation also occur in the axes of ventralised leaves in phan single mutants and in phan hb double mutants. In Arabidopsis mutants loss or gain of dorsal fate from organs can result in loss or gain of meristems in their axes (McConnell and Barton, 1995; McConnell and Barton, 1998; Siegfried et al., 1999), suggesting either that dorsal fate is necessary to promote axillary meristem formation or that both are specified by the same process. Similarly, loss of axillary meristems in phan and phan hb mutants may be a secondary consequence of defective dorsal fate. Because floral meristems in Antirrhinum are produced in the axes of leaf-like bracts, reduced flower formation in phan hb double mutants...
may reflect a similar requirement for dorsal bract identity in floral meristem formation, and dorsal identity in other floral organs for subsequent maintenance of the floral meristem.

The role of Phan and Hb in dorsoventral asymmetry of lateral organs

The hb mutation enhances the effects of phan mutations on dorsal fate in leaves, suggesting that Hb is additionally involved in this process. It also clarifies the role of Phan in cotyledons that are ventralised in phan hb double mutants, but not in phan single mutants, suggesting that both Phan and Hb act during embryogenesis to specify dorsal cotyledon fate. Because flowers of phan hb double mutants are always incomplete they do not reveal a role for Hb in dorsoventral asymmetry of floral organs or additional roles of Phan in flowers.

Phan is expressed in organs, therefore its role in dorsal organ fate might be cell-autonomous. Evidence from other species implies that non cell-autonomous signals within the SAM are also required to specify dorsal organ fate (e.g. Sussex, 1951). Genetic evidence does not suggest whether Hb might act in cell-cell signalling within the SAM or cell-autonomously in organs.

The role of Hb in dorsoventral asymmetry of flowers

Hb is required for normal lateral growth of flower petals and to promote their asymmetry. Its role in asymmetry appears not to be shared with Phan because asymmetry defects are seen in hb single mutants and not in phan single mutants. Neither does asymmetry appear to involve activity of the CSP, because wild-type flowers develop normally at low temperatures. However, Phan and the CSP are required for normal lateral growth of petal lobes (ventralised lobes of phan mutants are narrower than wild type). Therefore Phan and Hb may overlap in function to control lateral growth, although any redundancy is obscured in phan hb double mutants because they fail to produce recognisable petals.

The hb mutation causes the upper parts of each petal to resemble its lower parts, suggesting that Hb is required to specify upper fate in all petals. The Dich gene has a similar role in dorsal petals (Luo et al., 1999). dich hb double mutants show an additive phenotype, suggesting that Hb is needed to promote petal asymmetry by a different mechanism to Dich.

In addition to Dich, floral asymmetry requires interaction between the dorsalising genes Cyc and Rad and the ventralising activity of Div (Almeida et al., 1997; Carpenter and Coen, 1990; Luo et al., 1996; Luo et al., 1999). Each of these genes influences the development of more than one petal type – in cyc mutants, for example, the dorsal petal assumes lateral characteristics and the lateral petal assumes ventral identity. In contrast, the hb mutation affects asymmetry of all petals, but does not change their identities, suggesting that Cyc, Rad and Div have roles in both petal identity and internal asymmetry whereas Hb functions only in asymmetry. The additive phenotypes observed for hb and floral asymmetry mutations suggest that Hb does not act in the same process as Cyc, Dich or Rad and therefore is not regulated by these asymmetry genes. However, Hb is needed in the upper part of each petal and therefore Hb activity, or the requirement for it, appears to respond to dorsoventral asymmetry in the flower. Antirrhinum flowers develop from groups of initial cells within the flanks for the inflorescence SAM in a similar way to lateral organs. Because Hb is involved in dorsal fate of lateral organs, one possibility is that it promotes dorsal fate in developing flowers by the same mechanism.

The hb mutation results in an Antirrhinum flower that retains different petal identities along its dorsoventral axis but in which asymmetry of individual petals is significantly reduced. Such a flower is considered typical of more basal relatives of Antirrhinum (discussed by Luo et al., 1999). Therefore evolution of asymmetric petals might have involved an extension of the role of genes such as Hb to specify asymmetry in petals in addition to their ancestral roles in asymmetry of lateral organs.

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pleiotropically in Arabidopsis development and has overlapping functions with the ARGONAUTE1 gene. Development 126, 469-481.


