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The *PHANTASTICA* Gene Encodes a MYB Transcription Factor Involved in Growth and Dorsoventrality of Lateral Organs in *Antirrhinum*

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Summary

The organs of a higher plant show two fundamental axes of asymmetry: proximodistal and dorsoventral. Dorsoventrality in leaves, bracts, and petal lobes of *Antirrhinum majus* requires activity of the *PHANTASTICA* (*PHAN*) gene. Conditional mutants revealed that *PHAN* is also required for earlier elaboration of the proximodistal axis. *PHAN* was isolated and shown to encode a MYB transcription factor homolog. *PHAN* mRNA is first detected in organ initials before primordium initiation. The structure and expression pattern of *PHAN*, together with its requirement in two key features of organ development, are consistent with a role in specifying lateral organ identity as distinct from that of the stem or meristem. *PHAN* also appears to maintain meristem activity in a non-cell-autonomous manner.

Introduction

The above-ground part of a flowering plant consists of a series of organs born on continuous stem axes. A plant produces several different organ types in a defined sequence during its lifetime—leaves during vegetative growth and various floral organs later in development. This production of repeated structures with different identities can be compared to the segmentation pattern of insects. In *Drosophila*, different combinations of segment identity gene functions specify differences between segments, while shared segment polarity gene functions are responsible for similarities between them. Unlike plant organs, however, the segments of *Drosophila* arise synchronously by subdivision of the embryo rather than sequentially. In plants, much is known about genes involved in establishing the differences between organs (reviewed by Weigel and Meyerowitz, 1994), whereas little is known of the basis of similarities between organs.

The chief similarity between the organs of a higher plant is that they show two major axes of asymmetry. First, they exhibit a proximodistal axis lateral to the stem and are therefore collectively termed lateral organs (although organs may appear to form directly from the apex of the floral meristem as with carpel development in some species). The proximodistal axis becomes apparent as groups of organ initial cells on the flanks of the meristem form a primordium with a novel direction of growth. Morphological differences may be elaborated later along this proximodistal axis, for example, between the broad distal leaf blade and the narrower petiole (stalk) proximal to the stem. Second, lateral organs typically show dorsoventral asymmetry. Most are flattened in a plane perpendicular to their dorsoventral axis. The developmental stage at which this flattening becomes apparent varies between species. In many monocots, such as maize, it can be first seen when primordia emerge from the apical meristem, whereas in other species, proliferation may lead to flattening only after primordial emergence (e.g., Poethig and Sussex, 1985). Dorsoventral asymmetry is further apparent in the pattern of cell types within organs. In most organs, the upper (adaxial) surface is often distinct from the lower (abaxial), and dorsoventral differences may also be seen in internal tissues. In contrast to lateral organs, the main stems of most flowering plants are radially symmetrical.

One working hypothesis is that the two axes of lateral organs—proximodistal and dorsoventral—may be the result of genetic functions that are common to all organs. Because these characteristics are apparent from early in organ development, it is likely that the corresponding genetic functions are expressed in groups of initial cells that have assumed lateral organ identity at, or before, the time that they are visible as primordia.

What commits meristem cells to a lateral organ fate and therefore to elaboration of proximodistal and dorsoventral axes remains poorly understood. At least two genes required for normal meristem function are expressed in domains which suggest that they are involved in a meristematic prepattern distinguishing organ initials from the remainder of the shoot apical meristem (SAM). The homeobox gene, *SHOOT MERISTEMLESS* (*STM*), is required for the formation and maintenance of the SAM in *Arabidopsis* and is transcribed in cells of the apical meristem and internode initials (Clark et al., 1996; Long et al., 1996). Absence of *STM* expression from groups of cells flanking the SAM is the earliest known marker for lateral organ initials. In Petunia, the *No apical meristem* gene is expressed around the boundary between lateral organ initials and the SAM and is required for SAM formation (Souer et al., 1996). However, no gene has yet been identified that shows expression confined to the initials of all lateral organs and could therefore be a potential determinant of lateral organ identity.

We have previously identified a requirement for the *PHANTASTICA* (*PHAN*) gene in one lateral organ characteristic—dorsoventrality—in *Antirrhinum* (Waites and Hudson, 1995). In phan mutants, tissues associated with the dorsal part of the wild-type leaf can be replaced by tissues with ventral characteristics, suggesting that *PHAN* is required for identity of dorsal leaf initial cells. A relationship between *PHAN*-dependent dorsal cell identity and flattening of the leaf blade was also suggested, based on the appearance of phan mutant leaves that were mosaics of dorsal and ventral tissues. In all

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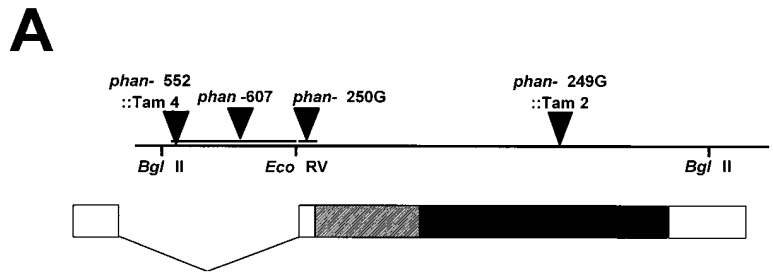

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Figure 1. Structure of the *PHAN* Locus

(A) Map of the *PHAN* genomic region. The insertion sites of *Tam4* in *phan*-552 and of *Tam2* in *phan*-249G are shown by black triangles (not to scale) on the restriction map. The regions in which *phan*-607 and *phan*-250G carry insertions are represented by lines with triangles above the map. Boxes below the restriction map represent exons. White boxes denote untranslated exon sequences, and the stippled box, the region encoding the conserved MYB domain.

(B) The amino acid sequence of PHAN is shown below its nucleotide sequence. The 5′ end of the cDNA sequence was obtained by RACE and is therefore assumed to represent the longest transcript. A second transcription initiation site at position 25 is indicated by an arrowhead. Splicing of an intron in the 5′UTR can occur at one of two donor sites, separated by the sequence shown in lower case. The two MYB repeats of the PHAN protein are underlined, and the novel C-terminal region encoded by the *phan*-249G allele, as the result of *Tam2* insertion, is shown in italics below the wild-type amino acid sequence.

cases, outgrowths resembling ectopic leaf blades were formed at the junctions between dorsal and ectopic ventral tissues. This indicated that interaction between dorsal and ventral cells might be responsible for outgrowth of the leaf blade in wild type in an axis perpendicular to the proximodistal axis of the primordium. *PHAN* also appeared to be required for dorsoventrality of bracts and petal lobes, but not for normal development of other lateral organs.

To examine further the function of *PHAN* in lateral organ development, we exploited the cold sensitivity of the phan mutant phenotype. This revealed an additional requirement for *PHAN* in proximodistal growth and patterning of lateral organ primordia. We have also isolated *PHAN* and shown that it encodes a MYB-related transcription factor. Expression of *PHAN* is specific to the initials and early primordia of all lateral organs. It is expressed throughout each primordium, indicating that spatially specific expression of *PHAN* within the primordium is not responsible for the establishment of proximodistal or dorsoventral axes. Rather, *PHAN* may interact with other genes that have spatially restricted expression patterns. The biochemical function, expression pattern, and requirement for *PHAN* in elaboration of proximodistal and dorsoventral organ axes are consistent with a role in the determination of lateral organ identity as distinct from that of the meristem and stem. We have also shown a requirement for *PHAN* in maintaining the activity of apical stem cell populations, which suggests that determination of lateral organ identity is essential for function of the apical meristem in higher plants.

Results

Isolation of the *PHANTASTICA* Gene

The *PHANTASTICA* (*PHAN*) locus was defined by four recessive mutations that disrupted development of leaves, bracts, and petal lobes. One mutation, *phan*-607, arose in a transposon mutagenesis program (Carpenter and Coen, 1990) and was shown to be allelic with two classical mutations, *phan*-249G and *phan*-250G (Baur, 1926; Stubbe, 1932). Plants homozygous for *phan*-607 gave rise to one wild-type plant among ~500 mutant progeny, suggesting that the mutation was caused by a transposon which could excise from the locus at low frequency to restore a functional *PHAN* allele. A further mutant allele, *phan*-552, was identified in a targeted transposon mutagenesis experiment (Waites and Hudson, 1995) and exploited to clone the locus. The mutation had been obtained by crossing homozygous *phan*-249G mutants to the wild-type line JI.75, which carries active transposons in its genetic background. A single phan mutant was identified among ~18,000 wild-type F1 progeny and assumed to be heterozygous for *phan*-249G and a newly mutated allele, *phan*-552. Southern hybridization revealed a copy of the *Tam4* transposon in the *phan*-552/*phan*-249G mutant, as a 6.3 kb BglII fragment that was not present in eight of its wild-type siblings. To test whether this copy of *Tam4* was responsible for the *phan*-552 mutation, the mutant was back-crossed to its *phan*-249G parent. A small proportion of the progeny (42 plants in 13 independent families) had wild-type phenotype, indicating that they carried *PHAN*$^+$ revertant alleles. About 50% of the mutant progeny were found to have inherited the copy of *Tam4*—a frequency expected from segregation of the *phan*-552 allele in gametes of the *phan*-552/*phan*-249G parent—whereas all 42 of their PHAN$^+$ revertant siblings lacked it. These results suggested that the *Tam4* insertion was responsible for the *phan*-552 mutation. The copy of *Tam4* and sequences flanking it were cloned (see the Experimental Procedures), and sequence analysis revealed that *Tam4* had inserted near to the end of a wild-type BglII fragment of 1.6 kb. Plants carrying the three other *phan* mutations also showed polymorphisms in this fragment consistent with different DNA insertions (Figure 1A). In the case of *phan*-607, transposon excision was correlated with phenotypic reversion to wild type. Together, these results strongly suggested that at least part of the *PHAN* gene was contained within the cloned DNA. The sequence flanking *Tam4* contained a single long open reading frame, and comparisons of wild-type genomic and cDNA clones indicated that this represented the entire PHAN protein coding region. An intron was present within the 5′ untranslated region, which began at one of two splice donor sequences 192 or 198 bp downstream of the major transcript initiation site and ended 49 bp upstream of the translation initiation codon (Figure 1B). The *Tam4* transposon in *phan*-552 was therefore present within this intron, 351 bp upstream of the splice acceptor.

The Structures of *phan* Alleles Correlate with Their Phenotypic Severity

The four *phan* mutations differed in their effects on petal development. The petal lobes of *phan*-250G mutants were consistently reduced to ventralized needles whereas the other *phan* mutations allowed development of petal lobes to increasing degrees, in the order *phan*-250G < *phan*-249G < *phan*-607 < *phan*-552. Weaker mutations were found to be dominant to stronger ones, suggesting that the weak and intermediate mutant alleles conferred reduced *PHAN* activity (hypomorphs). The structure and expression of the mutant alleles was therefore characterized further.

The weakest alleles, *phan*-607 and *phan*-552, contained insertions within the intron and were able to encode *PHAN* mRNA of wild-type size at reduced abundance (data not shown), suggesting that transposon sequences could be removed by splicing of the intron to allow production of a functional transcript. The intermediate allele, *phan*-249G, was found to contain a stable insertion of the *Tam2* transposon within the *PHAN* coding region (Figure 1A), which was flanked at its upstream end by an additional 2 bp (TC), suggestive of an aberrant insertion or abortive transposition event involving only this end of *Tam2*. As a result, the *phan*-249G allele had the potential to encode a novel protein in which the 40 C-terminal residues of PHAN were replaced by 34 amino acids encoded by *Tam2* (Figure 1B). Because the *phan*-249G allele conditioned an intermediate mutant phenotype, the novel protein was assumed to be expressed and to be at least partially functional. The strongest mutant allele, *phan*-250G, contained a stable insertion of about 4.5 kb between the *PHAN* splice acceptor site and start of translation (Figure 1A). No *PHAN* mRNA could be detected in vegetative or inflorescence tissues of plants homozygous for this mutation (Figure 3E), suggesting that the insertion prevented production of a functional *PHAN* transcript and therefore that *phan*-250G was a null allele.

The four *phan* mutations conditioned nearly identical vegetative phenotypes. The leaves produced early in development typically had laminae with patches of ectopic ventral tissues. Late leaves and bracts were usually reduced to ventralized needles, and intermediate leaves were either narrower than wild type, with laminae arising from more dorsal positions, or mosaics of normal and ventralized tissues. Although all the *phan* mutant alleles contained transposons or DNA insertions suggestive of transposons, over 50,000 progeny from each of the *phan*-249G and *phan*-250G mutants contained no wild-type revertants, suggesting that the mutations in these lines were genetically stable. Therefore, the variability in mutant leaf morphology did not appear likely to result from restoration of *PHAN* activity on somatic transposon excision; nor could the variability be attributed to residual *PHAN* activity in mutants because it was shown by plants homozygous for the potential null allele, *phan*-250G. It therefore seemed likely to reflect the activity of independently expressed factors that could partially substitute for *PHAN* in developing leaves.

PHAN Encodes a MYB-Related Transcription Factor

PHAN had the potential to encode a 42.4 kDa protein of 357 amino acids (Figure 1B). Imperfect tandem repeats of 56 and 51 amino acids at the N terminus of the PHAN protein showed significant similarity to the motifs

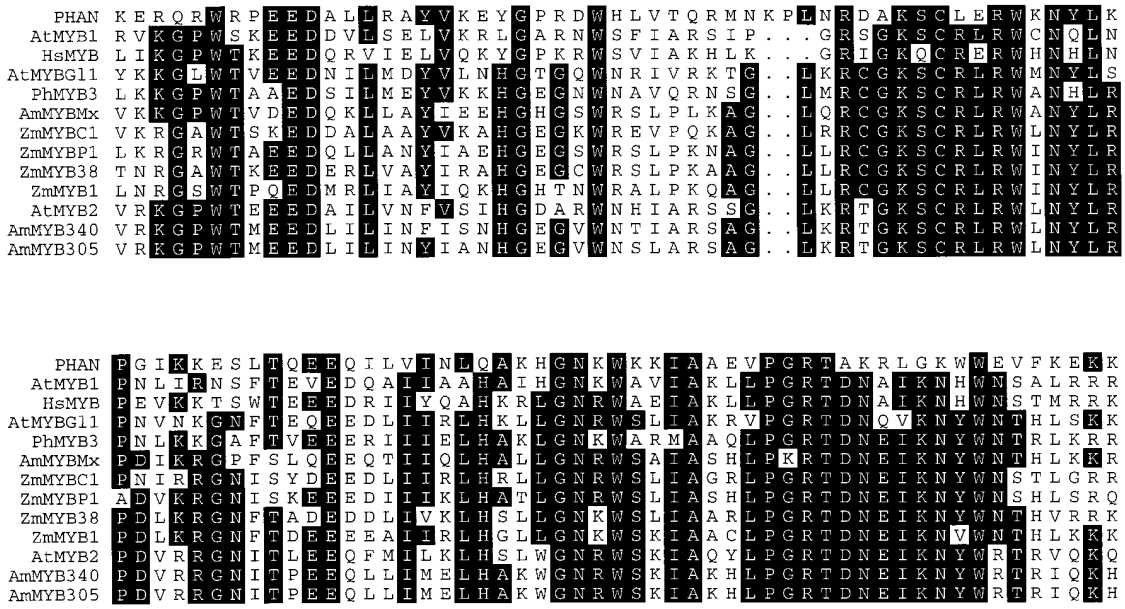

Figure 2. Sequences Similar to PHAN

The two imperfect amino acid repeats of PHAN are shown aligned with those of a number of MYB transcription factors that have defined functions and/or demonstrated DNA binding activities. Residues that are identical in the majority of these proteins are boxed. The nomenclature for MYB proteins is that of Martin and Paz Ares (1997).

present in 1–3 copies close to the N termini of all known MYB-related transcription factors (Figure 2). Although PHAN contained two of these repeats, in common with most plant MYB proteins, it showed a high degree of similarity to the product of only one gene, of unknown function, induced on root-knot nematode infection of tomato (Bird and Wilson, 1994). It differed from other MYB proteins in three obvious respects: the C-terminal region of the second repeat (residues 93–102) showed little sequence conservation, PHAN had only one amino acid upstream of the first repeat, and the first repeat was 2 or 3 amino acids longer than that of other MYB proteins (Figure 2).

Downstream of the conserved MYB domain, PHAN showed no sequence similarity to any characterized protein, except for the product of a related gene of unknown function from tomato. However, in common with many MYB proteins, the C-terminal region contained a high proportion of negatively charged residues.

PHAN mRNA Is Restricted to Lateral Organ Initials and Primordia

Expression of *PHAN* was examined in wild-type seedlings. In the early phase of vegetative growth, wild-type plants produce a pair of leaves at each node from groups of initials at opposite sides of the shoot apical meristem (SAM) and at ~90° to the pair of leaf primordia at the previous node (decussate phyllotaxy). By convention, nodes with emerged leaves are numbered from the top of the shoot downward, so that the pair of primordia to have emerged most recently from the apex are termed P_1. Cells within the SAM that will form the next pair of primordia are designated P_0, and initials of subsequent primordia, I_1, I_2, and so forth. The primordia of *phan* mutant leaves differed from those of wild type from about the P_1 stage, by their more radial appearance, indicating that *PHAN* is required for dorsoventrality early in leaf development (Waites and Hudson, 1995).

Digoxigenin-labeled antisense RNA was transcribed from part of the *PHAN* gene downstream of the conserved *MYB* region and used to probe sections of wild-type seedlings. *PHAN* mRNA expression was detected in two opposite domains of the meristem corresponding to the positions of P_0 initials and in newly emerged P_1 primordia (Figures 3A and 3B). To examine whether *PHAN* mRNA was restricted to leaf initial cells, its domain of expression was compared to that of *AmSTM*, the *Antirrhinum* homolog of the *Arabidopsis SHOOT MERISTEMLESS* gene (see the Experimental Procedures). In *Arabidopsis*, *STM* is expressed in cells of the SAM, but not in leaf primordia, and loss of expression from leaf initials provides the earliest known marker of leaf fate (Long et al., 1996). In adjacent sections of the wild-type *Antirrhinum* SAM, the domains of *PHAN* and *AmSTM* expression appeared to be complementary (Figures 3C and 3D). *PHAN* mRNA was restricted to leaf initial cells and uniformly distributed throughout them. *PHAN* mRNA expression persisted in developing leaves but was undetectable after late stage P_3. By this stage, the wild-type leaf showed dorsoventral differences in cell type (e.g., trichomes produced from only the dorsal surface) and marked dorsoventral flattening (Figures 3A and 3B).

Because *PHAN* was also required for normal development of bracts and petal lobes, its expression was characterized in wild-type inflorescences. In contrast to young vegetative shoots, the inflorescence produces

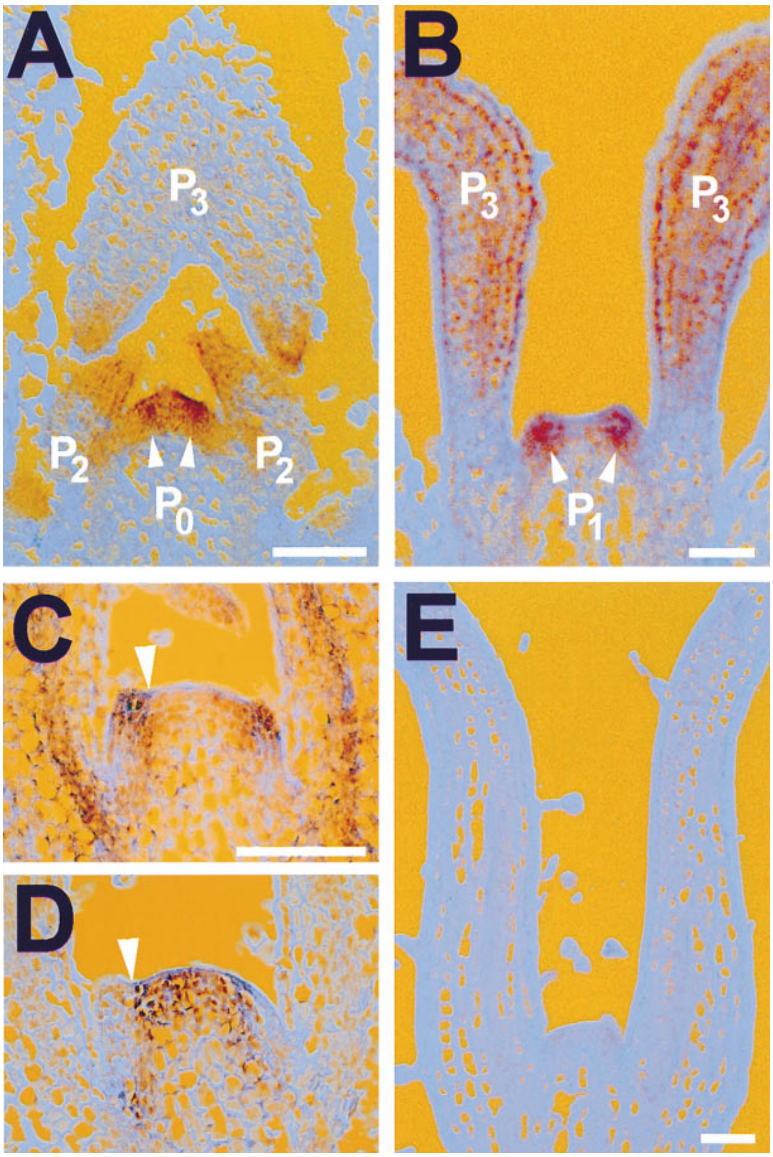

Figure 3. Vegetative Apices Probed with *PHAN* or *AmSTM*

Longitudinal sections of wild-type shoot tips passing through leaf initials and primordia at different developmental stages (A and B) were probed with *PHAN*. Hybridization of adjacent sections of wild-type apices with *PHAN* (C) or *AmSTM* (D) probes revealed that their expression patterns were complementary (see arrowheads). No *PHAN* mRNA could be detected in apices of the *phan*-250G mutant (E). Scale bar is 100 μm. P_0, etc., denotes the developmental ages of leaf initials and primordia (see text for details).

bracts singly in a spiral arrangement, so that each bract primordium emerges from the apex above P_4 and P_6 primordia initiated earlier. *PHAN* mRNA was detected in domains of the inflorescence meristem corresponding to the position of bract initials from stage I_3 onward (Figure 4A). Its expression was compared with that of *FLORICAULA* (*FLO*), which is first detected in bract initials from the P_0 stage, providing an early marker for bract fate, and subsequently in floral meristems subtended by existing bract primordia (Coen et al., 1990). Hybridization to adjacent sections showed that *PHAN* expression began earlier than that of *FLO* (Figure 4B) and revealed that the domain of *PHAN* mRNA expression in older, P_0, bract initials coincided with that of *FLO* (data not shown). As in leaves, *PHAN* expression persisted in developing bracts becoming undetectable by about stage P_{10}.

During development of wild-type flowers, *PHAN* mRNA was initially absent from floral meristem precursors within the inflorescence meristem and from newly initiated floral meristems, marked by uniform expression of *FLO* (Figures 4A and 4B). It was subsequently detected in sepal initials slightly before the stage that *FLO* expression became limited to the same domain (Figures 4C and 4D). At about the floritypic stage, when expression of the floral homeotic genes *DEFICIENS* (*DEF*) and *PLENA* (*PLE*) became established in partially overlapping domains of the floral meristem, *PHAN* mRNA declined slightly in sepal primordia and appeared in petal initials that are marked by expression of *FLO* but not *PLE* and in stamen initials that express *PLE* but not *FLO* (Figures 4E–4F). The timing of floral homeotic gene expression has suggested that petal and stamen identities are determined at approximately the same time (Bradley et al., 1993), and primordia of both organ types initiate almost simultaneously (Carpenter et al., 1995). In flowers at later stages of development, *PHAN* expression was detected in stamen and carpel initials and

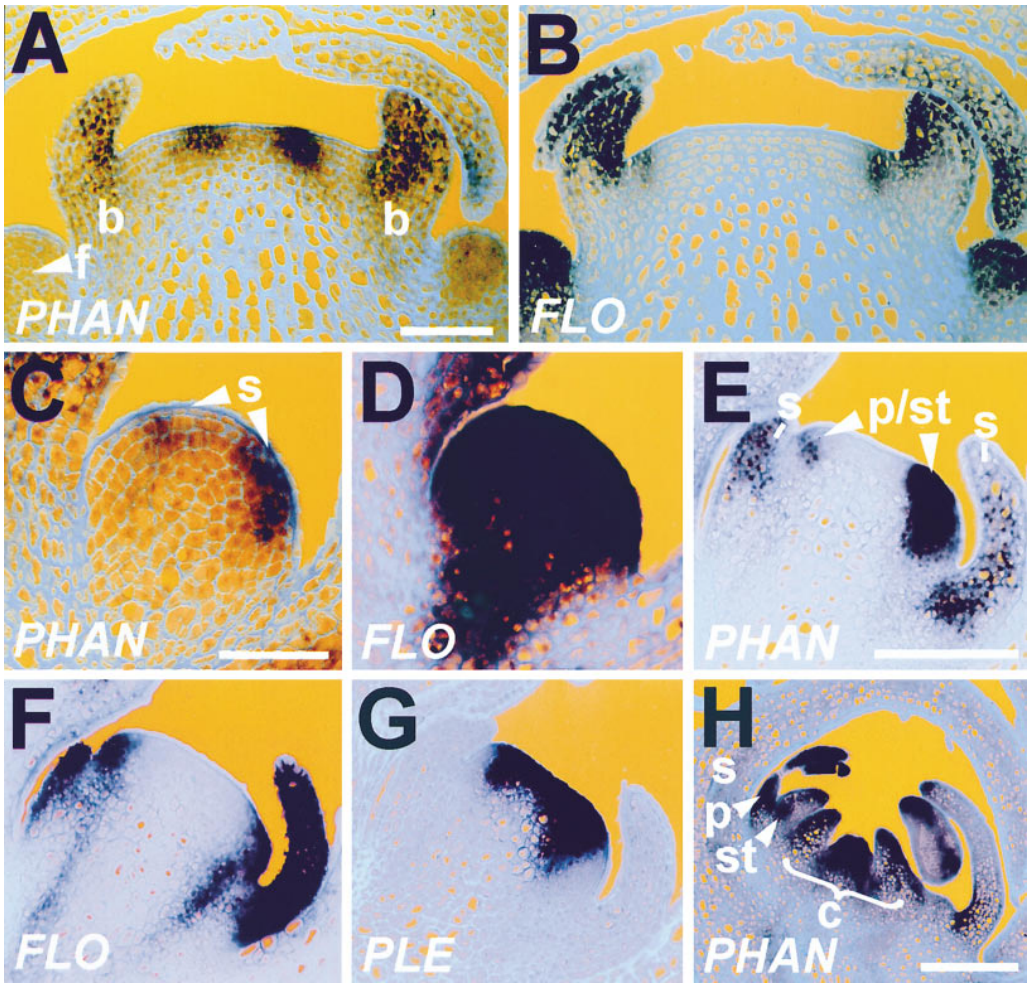

Figure 4. *PHAN* mRNA Expression in Inflorescence and Floral Meristems

Adjacent longitudinal sections of wild-type inflorescence apices were probed with (A) *phan* or (B) *FLO*. In serial sections of early floral meristems, *PHAN* mRNA was detected in sepal initials (C) before expression of *FLO* became confined to the same domain (D). The pattern of *PHAN* transcription in wild-type floral meristems at about the floritypic stage (E) was compared in serial sections to that of *FLO* (F) and *PLE* (G) and characterized in wild-type flowers at a later stage in development (H). Scale bars represent 100 μm in (A), (E), and (H) and 50 μm in (C). Young floral meristems are indicated by f, and the initials or primordia of bracts, sepals, petals, stamens, or carpels are indicated by b, s, p, st, and c, respectively.

primordia (Figure 4H). *PHAN* therefore appeared to show an equivalent pattern of expression in all lateral organs, although phan mutant phenotypes indicated that it was required only for development of normal leaves, bracts, and petal lobes.

PHAN Is Needed for Lateral Organ Initiation and Meristem Function

The phan mutant phenotype was almost completely suppressed in plants grown at 25°C, and enhanced at lower temperatures. At 17°C, all except the first two pairs of leaves were completely ventralized. Because this temperature response was shown by all mutants, including those carrying the likely null allele *phan*-250G, it was assumed to reflect the temperature sensitivity of functions that reduced the requirement for *PHAN* at higher temperatures. We therefore tested whether a greater requirement for *PHAN* might be imposed by growing plants at an even lower temperature. At 15°C, *phan* mutant seedlings arrested development after producing only one or two pairs of leaves (Figure 5A). Primordia of the subsequent pair of leaves either failed to initiate or arrested development at the P_1 stage (Figure 5B). The apical meristems of these plants also ceased growth and appeared flatter and less organized that those of wild-type plants (compare Figures 5B and 5C). Meristems that had formed in the axils of existing leaves were affected in a similar way. In contrast, wild-type plants were able to grow normally and to flower at 15°C. (Figure 5D). These results indicated that *PHAN* had additional roles in initiation and proximodistal outgrowth of leaf primordia, and in maintaining activity of shoot apical meristems. Reducing the requirement for *PHAN*, by returning plants to the permissive temperature, allowed leaf initiation and meristematic growth to resume even after 6 weeks at 15°C (Figure 5E). Primordia initiated upon shifting to the permissive temperature formed normally shaped leaves, although these were irregularly

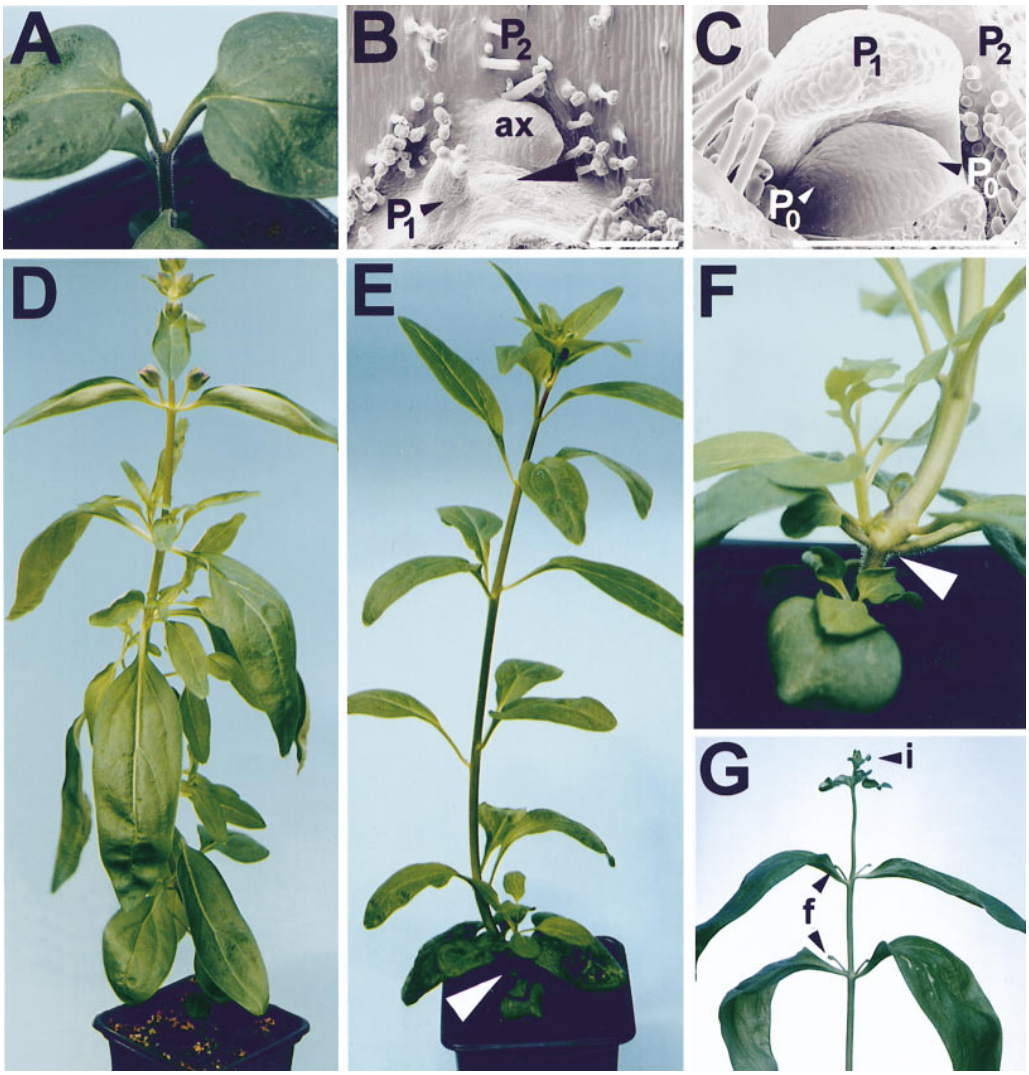

Figure 5. Temperature-Sensitive Effects of *phan* Mutations on Organ Initiation and Meristem Function

(A) A *phan*-607 mutant seedling arrested in growth after germination at the nonpermissive temperature of 15°C. (B) The apical region of this plant compared to the apex (C) of a wild-type plant (D) of a similar age under the same conditions. A secondary meristem in a leaf axil is indicated by ax, and the arrowhead points to the region corresponding in position to the apical meristem of wild type. Shifting *phan* mutants from 15°C to the permissive temperature of 25°C allowed meristem function to resume (E), although initial leaves were aberrantly positioned on the stem axis (F). In this case, the major shoot produced upon shifting to the permissive temperature (above the white arrowheads) arose from an arrested axillary meristem; in other cases, it was produced by a resumption in growth of the SAM. Shifting plants to the nonpermissive temperature later in development (G) inhibited inflorescence (i) and floral meristem (f) development and initiation and growth of floral organ primordia. Scale bars denote 100 μm.

spaced on the stem axis for several nodes (Figure 5F), suggesting an involvement of *PHAN* in phyllotaxy.

To investigate when *PHAN* was required for leaf initiation, phan mutants were grown initially at 25°C and then transferred to the restrictive temperature of 15°C. The developmental stage of each plant at the time of transfer was estimated from the length of existing leaves (see the Experimental Procedures). In a significant proportion of plants (61%), the last leaves to be produced were those derived from P_1 primordia (15%) or P_0 initials (46%) at the time of the temperature shift. The requirement for *PHAN* in the emergence of these primordia was therefore consistent with the earliest observed stages of *PHAN* mRNA expression. The remaining 39% of plants showed a more delayed response to the temperature shift, initiating an additional one or two pairs of leaves before meristem arrest. Ventralized leaves were observed only rarely, and leaves that had been older than stage P_1 at the time of the temperature shift were almost always able to develop normally. Similarly, phan mutant meristems that had arrested at the restrictive temperature initiated only dorsoventrally flattened leaves on return to 25°C. This suggested that the requirement for *PHAN* in dorsoventrality did not extend beyond that in primordium emergence and early proximodistal growth.

The potential role of *PHAN* expression in bract and floral organ primordia was examined by transferring phan mutants to the restrictive temperature after the

transition to flowering. Initiation of bract primordia and activity of inflorescence meristems were inhibited. Furthermore, floral meristems either failed to initiate organ primordia or arrested after production of one or more whorls of rudimentary floral organs (Figure 5G). These results suggested that there was a similar requirement for *PHAN* in initiation of all lateral organ primordia and in maintaining the normal activity of all apical meristems. The effects of the temperature shift on floral development occurred several nodes below those on bract initiation, consistent with the delay of about six nodes between initiation of a bract primordium and initiation of the floral organs from the meristem in the axil of that bract (Carpenter et al., 1995).

Discussion

We have demonstrated that *PHAN* is required for elaboration of the proximodistal axis of all lateral organs, for dorsoventral asymmetry in a subset of organs, and to maintain the activity of apical meristems. The *PHAN* gene encodes a MYB-related transcription factor. MYB proteins are characterized by 1–3 N-terminal copies of a conserved sequence (the MYB repeat) that function in binding target DNA (Tanikawa et al., 1993), and C-terminal terminal regions that show little sequence conservation. Higher plant genomes encode numerous MYB proteins, and those for which functions have been determined regulate diverse developmental and metabolic processes (Martin and Paz Ares, 1997). The PHAN protein contains two repeats, in common with most plant MYBs. However, these are unconventional in several respects. The first repeat is 2 or 3 amino acids longer than other MYB proteins and has only one amino acid upstream of it, rather than a short leader sequence. The C-terminal region of the second repeat, which is highly conserved in other proteins and implicated in DNA binding specificity, is poorly conserved in PHAN. The *Antirrhinum* genome has the capacity to encode at least one additional PHAN-like protein, which shares these unconventional characters and also shows conservation with PHAN in its C-terminal region (unpublished data). These results suggest that *PHAN* is a member of a small subfamily of *MYB* genes which may be the result of an ancient divergence. The only other characterized member of this subfamily is expressed following root-knot nematode infection of tomato roots (Bird and Wilson, 1994). Infection induces differentiation of a novel cell type and ectopic expression of a number of genes (Opperman and Conkling, 1994), and the functional significance of *PHAN*-like gene expression in this process therefore remains unclear.

PHAN Is Required for Elaboration of a Proximodistal Axis in All Lateral Organs

The phan mutant phenotype is more severe at lower temperatures. This cold sensitivity is shared by all mutants, including those carrying a likely null allele, suggesting that it reflects the response to temperature of an independent factor which overlaps in function with *PHAN*. Null mutations that reveal the inherent temperature sensitivity of redundant pathways have previously been identified in *C. elegans* and *Drosophila* (e.g., Strome et al., 1995). The requirement for *PHAN* at the permissive temperature of 25°C appears minimal because all phan mutants closely resemble wild-type plants. A greater requirement, induced by growing mutant plants at the restrictive temperature of 15°C, reveals that *PHAN* is needed for all lateral organs to elaborate a proximodistal axis—primordia either fail to emerge or cease proximodistal growth at this temperature. In leaves, the requirement for *PHAN* extends from at least the stage immediately before appearance of primordia (P_0) into the stage after (P_1), because shifting mutant plants from 25°C to the restrictive temperature can prevent initiation from P_0 initials and growth of P_1 primordia, whereas older leaves remain unaffected. The role of *PHAN* function in primordium initiation and subsequent proximodistal growth coincides with its pattern of early expression as *PHAN* mRNA is confined to lateral organ initials and primordia. In leaves, it is first detected before primordia emergence in P_0 initials and persists in primordia until stages P_3–P_4.

PHAN Is Needed for Dorsoventral Asymmetry in a Subset of Lateral Organs

In addition to forming a proximodistal axis, lateral organs typically elaborate dorsoventral asymmetry. The temperature sensitivity of phan mutants allows the role of the *PHAN* function in these two processes to be partially separated. At the intermediate temperature of 17°C, the requirement for *PHAN* is reduced to a level sufficient for initiation of all organ primordia, although in leaves, bracts, and petal lobes it remains insufficient for determination of dorsal cell identity because these organs in phan mutants typically show no dorsoventral asymmetry and consist only of ventral cell types. Lack of dorsoventral asymmetry, including lateral growth, is consistent with the view that lateral growth results from *PHAN*-dependent dorsal cell identity. However, the alternative—that *PHAN* acts to specify lateral growth and that dorsal identity is a consequence of this—cannot be ruled out. Although primordium emergence occurs at 17°C, other aspects of proximodistal axis elaboration are not fully restored. Ventralized organs show reduced proximodistal growth and are therefore shorter than wild type, and they also exhibit reduced proximodistal asymmetry—for example, leaves show no distinction between distal blade and proximal petiole (Waites and Hudson, 1995). At 17°C, other floral organs—sepals, stamens, and carpels—develop normally, suggesting that other genes act redundantly with *PHAN* in these organs to specify dorsal identity and proximodistal growth after primordium initiation. Consistent with this, we have identified a *PHAN*-like gene that is expressed in inflorescences (unpublished data) and might therefore fulfill this role. At the higher temperature of 20°C, leaves, bracts, and petal lobes of *phan* mutants typically consist of mosaics of normal and ventralized tissues. Such mosaicism is consistent with dorsal and ventral identities being mutually exclusive and clonally heritable. Reduced dorsal specification in phan mutants could allow cells to assume ventral identity, rather than an intermediate state, and to give rise to a clone of ectopic ventral tissue.

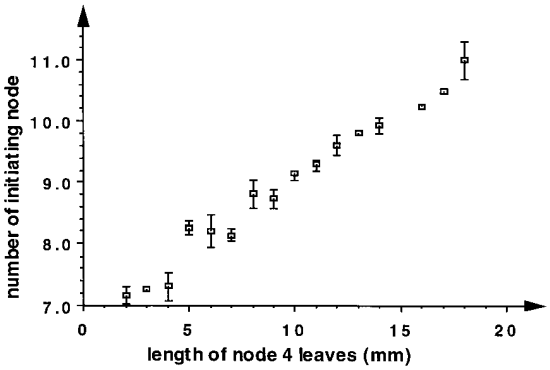

Figure 6. Estimating the Developmental Stage of the SAM from the Lengths of Existing Leaves

phan-607 mutants were grown at the permissive temperature of 25°C and the lengths of leaves at node 4 compared to the number of the node last initiated at the apex. The mean developmental stage of at least 15 plants at each leaf length was determined and plotted with standard errors.

Dorsoventral asymmetry only becomes apparent in *Antirrhinum* organs after they have begun to elaborate a proximodistal axis at primordium emergence. However, temperature shift experiments suggest that the requirement for *PHAN* function in dorsoventrality does not extend beyond the requirement in primordium initiation and outgrowth. Therefore, dorsoventrality may be specified within the shoot apical meristem (SAM), as previously suggested by surgical experiments in a number of species (Sussex, 1955; Snow and Snow, 1959; Hanawa, 1961). Determination of dorsoventrality in response to apical–basal polarity of the SAM would allow lateral organs to be oriented with reference to the stem axis.

Because *PHAN* is needed for the two key characteristics of lateral organs, its primary role may be to specify lateral organ identity as distinct from that of the meristem or stem. The site and timing of *PHAN* expression is also consistent with this role. Surgical experiments have suggested that the identity of leaves is irreversibly determined in P_0 initials (Snow and Snow, 1933), corresponding to the earliest stage of *PHAN* expression in *Antirrhinum*. Comparing the timing of *PHAN* with that of other genes, such as *FLO* and floral homeotic genes, which act as markers of lateral organ fate, suggests that it also has an early role in bract and floral organ development. That *PHAN* expression is limited to lateral organ initials implies that it interprets an existing prepattern. However, relatively little is known of the mechanisms that pattern the SAM and direct cells to lateral organ fates. Analysis of *Knotted1*-like homeobox genes, such as *STM* in *Arabidopis*, has provided some insights. *STM* is expressed in SAM cells, and its down-regulation in P_0 initials provides an early marker of leaf fate (Long et al., 1996). Furthermore, loss of *STM* expression may be sufficient for lateral organ identity because the meristems of weak stm mutants can terminate at their summits in production of ectopic lateral organs (Clark et al., 1996). However, loss of *STM* activity may not be sufficient for determination of organ fate in normal positions, because ectopic expression of *STM*-like genes of the *Knotted1* family in the lateral organ initials of a number of species does not prevent primordium initiation (e.g., Smith et al., 1992; Matsuoka et al., 1993; Lincoln et al., 1994). In *Antirrhinum*, expression of the *STM* homolog disappears from leaf initials at about the time that they begin to express *PHAN*. Therefore, one explanation is that *STM* acts as a negative regulator of *PHAN* expression and organ identity, or vice versa.

Requirement for *PHAN* in Meristem Maintenance

Apical and axillary meristems of phan mutants that have ceased initiating leaves at the restrictive temperature do not continue to produce leafless stem axes. They lose their characteristic structure and show no evidence of continuing cell growth or division. However, apical cells can remain viable and not undergo irreversible differentiation, because they can regain meristematic activity on return to the permissive temperature. Because *PHAN* mRNA is restricted to lateral organ initials but is required for activity of the meristem as a whole, this function is likely to involve a *PHAN*-dependent signal originating from organ initials or primordia. Although several other genes have been identified that are necessary for meristem activity and elaboration of organ axes (Medford et al., 1992; Talbert et al., 1995; Laux et al., 1996; Pickett et al., 1996), none affect only lateral organ emergence and dorsoventrality but not activity of the meristem. Similarly, few higher plant species appear capable of producing stem axes without at least rudimentary lateral organs. Therefore, definition of lateral organ identity may not simply involve partitioning of meristematic cells into a more determined fate but may itself be necessary for activity of the stem cell population within the meristem.

Experimental Procedures

Plants and Growth Conditions

The origins of *phan* mutants and their wild-type progenitors have been described previously (Waites and Hudson, 1995). To analyze the effects of temperature, wild-type and *phan* mutant plants were grown from seeds that had been surface-sterilized to encourage germination and sown singly in 8 cm pots of Levington M3 compost. Because germination of both wild-type and *phan* mutants is inhibited at 15°C, newly sown seeds were maintained at 20°C for 7 days and subsequently transferred to growth rooms at 15°C or 25°C, with a 16 hr photoperiod provided by illumination from metal halide lamps (100 μmol $m^{-2}s^{-1}$ of photosynthetically active quanta). Germination was not apparent at the time of transfer. To estimate the developmental stage of plants at the time of temperature downshifts, a correlation was sought between the lengths of leaves that had emerged from the apical bud and the number of the node being initiated at the apex, determined by dissection. The morphology of the youngest leaf primordia (whether they overtopped the SAM or whether there was an obvious crease between their dorsal surface and the SAM) was used to divide further the interval between emergence of successive nodes. For both phan mutant and wild-type plants grown at 25°C, the lengths of leaves at node 4 provided a reliable indication of the developmental stage of initiating primordia at nodes 7–10 (Figure 6). Temperature shifts were performed in situ by heating or cooling over a period of 2 hr to avoid potential effects of differences in lighting between growth rooms.

Microscopy and In Situ Hybridization

Scanning electron microscopy was carried out as described previously (Waites and Hudson, 1995). Material for in situ hybridization was fixed, sectioned, and hybridized to digoxigenin-labeled RNA

probes as described by Bradley et al. (1993). Templates for transcription of *PHAN*-specific probes were derived from positions 705–1413 or 995–1413 of the *PHAN* cDNA, downstream of the conserved *MYB* region, subcloned in either sense or antisense orientations relative to the T7 promoter of pBluescript II (Stratagene). Templates for transcription of *FLO*, *DEF*, and *PLE* probes have been described by Coen et al. (1990), Sommer et al. (1990), and Bradley et al. (1993), respectively, and were kindly provided by these authors. The *Antirrhinum* homolog of the *STM* gene, used as an in situ hybridization marker, was obtained by probing an *Antirrhinum* inflorescence cDNA library with an *STM* cDNA clone at low stringency. The *Antirrhinum* gene, *AmSTM*, had the potential to encode a protein that was more similar to the *STM* gene product (73% identity to the 270 C-terminal amino acids of STM) than the product of *STM* was to that of the most closely related *Arabidopsis* sequence, *KNAT1* (54% amino acid identity in the same region). In common with all KNOTTED1-like proteins, AmSTM showed little sequence conservation in its N-terminal region. These results suggested that *AmSTM* was the likely ortholog of *STM*. Probes for in situ hybridization were transcribed from the 5′-UTR and the region of *AmSTM* encoding the poorly conserved 106 N-terminal amino acids.

Cloning *PHAN*

To identify a transposon responsible for the *phan*-552 mutation, the *phan*-552/*phan*-249G heterozygote was back-crossed to its *phan*-249G mutant parent to produce 13 families, each derived from a single seed capsule. From each family, DNA was extracted from a pool of PHAN$^+$ revertants and from a pool of mutant siblings and used in low-stringency Southern hybridization with a 600 bp probe derived from the 3′ end of *Tam4* (Luo et al., 1991). This allowed detection of a 6.3 kb BglII fragment that was present only in pools of mutant plants. High-stringency Southern hybridization with probes specific to individual members of the CACTA transposon family revealed that the fragment carried a copy of *Tam4*. A size-fraction of BglII-digested DNA containing only this copy of *Tam4* was restriction mapped by Southern hybridization, revealing that the transposon had inserted close to one of the BglII sites and that the shorter flanking region contained an EcoRI site in addition to that present in *Tam4*. To facilitate cloning, BglII fragments of ~6.3 kb were blunt-ended, ligated to EcoRI adaptors, and then digested with EcoRI. The left and right ends of *Tam4* were cloned into the EcoRI site of λgt10 from appropriate size fractions. Sequences flanking the right end of *Tam4* were subsequently used as probes in isolation of wild-type *PHAN* clones from cDNA and genomic libraries.

The start of *PHAN* transcription was mapped by 5′ RACE analysis using the modifications of Frohman and Martin (1989). cDNA was synthesized from mRNA expressed in inflorescence apices or vegetative shoot tips using a primer complementary to positions 1339–1362 of the cDNA. It was homopolymer-tailed and amplified sequentially using primers from positions 694–677 and 489–471 of *PHAN*.

Restriction mapping of the *phan*-249G allele revealed that it carried a DNA insertion within the coding region. An EcoRI fragment extending from within the insertion to a site beyond the 3′ end of *PHAN* was cloned from size-fractionated DNA. Once sequence analysis had revealed that the insertion was a copy of *Tam2*, primers complementary to the other end of *Tam2* were used to amplify a region that included the upstream junction. Other parts of the *PHAN* coding region were amplified from *phan*-249G and sequenced to confirm that the allele carried no further mutations. The *phan*-250G allele was found to contain a DNA insertion 3′ to an EcoRV site 2–8 bp upstream of the *PHAN* splice acceptor. PCR using a primer in the *PHAN* coding region (positions 694–677) was able to amplify DNA from both wild-type and *phan*-250G mutants when used with a second primer beginning at the *PHAN* initiation codon (positions 188–206), but it was able to amplify only wild-type DNA when used with a primer from the intron, suggesting that the insertion in *phan*-250G lay between the splice acceptor sequence and *PHAN* protein coding region. The restriction map of the insertion did not correspond to that of any known transposon.

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