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The No Apical Meristem Gene of Petunia Is Required for Pattern Formation in Embryos and Flowers and Is Expressed at Meristem and Primordia Boundaries

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Summary

Petunia embryos carrying the *no apical meristem* (*nam*) mutation fail to develop a shoot apical meristem. Occasional shoots on *nam*⁻ seedlings bear flowers that develop ten instead of five primordia in the second whorl. Double mutants with the homeotic gene *green petals* show that *nam* acts independently of organ identity in whorl 2 and now also affects primordium number in whorl 3. The *nam* gene was isolated by transposon tagging. The encoded protein shares a conserved N-terminal domain with several other proteins of unknown function and thus represents a novel class of proteins. Strikingly, *nam* mRNA accumulates in cells at the boundaries of meristems and primordia. These data indicate a role for *nam* in determining positions of meristems and primordia.

Introduction

The starting point for development of higher plants is the fusion of the pollen cell with the egg cell to form the zygote. During embryo development, an apical–basal and a radial pattern are formed, determining the ultimate embryo body plan. The radial pattern consists of three concentric elements, from inside to outside: vascular tissue, ground tissue, and epidermis. The apical–basal pattern consists of the root meristem, the hypocotyl, and the two cotyledons flanking the shoot apical meristem (SAM). While the first three apical–basal pattern elements are already morphologically recognizable in heart-stage embryos, the SAM appears during the torpedo stage (Barton and Poethig, 1993; Jürgens et al., 1991; Mayer et al., 1991). Extensive mutagenesis experiments in Arabidopsis identified several mutants in which pattern formation is altered (Errampali et al., 1991; Feldmann, 1991; Goldberg et al., 1994; Jürgens, 1995; Jürgens et al., 1991; Mayer et al., 1991; West and Harada, 1993). Most of the apical–basal pattern mutants seem to have lost one or more pattern elements. For example, mutations in the *monopteros* gene cause the deletion of all basal structures such as root and hypocotyl, whereas *gurke* mutants delete both cotyledons and SAM (Mayer et al., 1991).

Mutants missing only the SAM have been described in several species, including Arabidopsis (Barton and Poethig, 1993; Jürgens et al., 1994), maize (Sheridan and Clark, 1993), and rice (Nagato et al., 1989). The *shoot meristemless* (*stm*) mutant of Arabidopsis fails to develop a SAM during embryogenesis, and normal shoots cannot be formed in vitro, suggesting that the *stm* gene controls both embryonic and adventitious SAM formation (Barton and Poethig, 1993). *stm* encodes a homeodomain protein and is predominantly expressed in the SAM and precursor cells, where it is thought to maintain cells in an undifferentiated state (Long et al., 1996). The other gene involved in apical–basal patterning that has been analyzed molecularly is *emb30/gnom*, encoding a protein with a 200 amino acid conserved domain (the Sec7 domain) (Shevell et al., 1994), of which the function is not well understood.

After germination, the SAM initially generates vegetative structures such as leaves, internodes, and axillary meristems. Under the influence of external and internal factors (Bernier, 1988), the vegetative meristem switches to an inflorescence meristem that generates bracts and floral meristems. Within each floral meristem, organ primordia are initiated at specific positions. In a second process, the identity of these organ primordia is specified, resulting in a flower that consists of four whorls occupied by sepals, petals, stamens, and carpels, respectively. Molecular genetic analyses show that organ identity is determined by the combinatorial action of a small set of genes, mostly encoding transcription factors with a common sequence motif, the MADS domain (Bowman et al., 1991; Coen and Meyerowitz, 1991; Haughn et al., 1995; Huijser et al., 1992; Schultz and Haughn, 1993; Schwarz-Sommer et al., 1990; Weigel and Meyerowitz, 1994). However, far less is known about the first process of floral development, which determines the position and number of organ primordia.

Here, we describe the *no apical meristem* (*nam*) mutant of Petunia and present the molecular characterization of this gene. The *nam* gene is required for SAM formation during embryogenesis. Analysis of mature plants that occasionally develop from *nam*⁻ seedlings shows that *nam* also plays a role in the positioning of organ primordia in the floral meristem. The *nam* gene product belongs to a novel class of proteins with unknown function. In situ mRNA localization shows that on many sites, *nam* expression marks the boundaries of initiating meristems and primordia.

Results

The *nam* Gene Product Is Required for Apical Meristem Formation During Embryogenesis

The Petunia line W138 contains more than 200 copies of the 284 bp transposable element *dTph1* (Gerats et al., 1990; Souer et al., 1995), which contributes to the high incidence of new mutations in progeny (Doodeman et al., 1984). In a random transposon–mutagenesis experiment, we screened thousands of W138 F2 families and identified one family, S227, that segregated for a mutation that arrested seedling development (Figure 1A). We called this mutation *nam*. In further crosses, this mutation segregated as a single recessive nuclear gene.

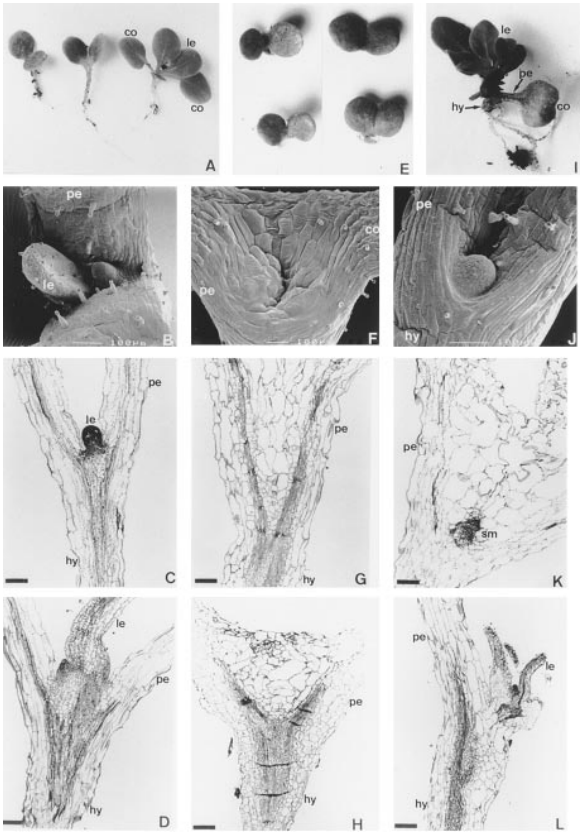

Figure 1. Phenotype of Wild-Type and *nam*⁻ Seedlings

(A) *nam*⁻ seedlings (left and middle) and wild-type seedling (right) 10 days after germination.

(B) SEM micrograph of wild-type seedling that has developed the first leaves (top view).

(C) Section through a young wild-type seedling. The SAM is located just above the branching vascular bundle and has generated a young leaf.

(D) Section through a wild-type seedling at later stage of development.

(E) Variability in cotyledon arrangement of *nam*⁻ seedlings.

(F) SEM micrograph of *nam*⁻ seedling containing large cells at the position where the SAM normally develops. Cotyledon cells can be recognized by their jigsaw shape.

(G) Section through a young *nam*⁻ seedling with large vacuolated cells between the petioles of the two cotyledons.

(H) Section through a *nam*⁻ seedling at a later stage in development. Note the absence of typical SAM cells.

(I) Escape shoot emerging from *nam*⁻ seedling. The meristem develops at its normal position where the petioles of the cotyledon branch.

(J) SEM micrograph of a *nam*⁻ seedling developing an escape shoot.

(K) Section through a young *nam*⁻ seedling. Note the cluster of meristematic cells that are surrounded by large vacuolated cells.

(L) Section through a *nam*⁻ seedling with escape shoot later in development. The escape SAM has already formed two leaves but is not properly connected to the vascular bundle in the hypocotyl.

(co, cotyledon; hy, hypocotyl; sm, shoot apical meristem; le, leaf. Scale bars in C, D, G, H, K, and L indicate 150 μm.)

In wild-type seedlings, the SAM appears as a dome of small cells between the petioles of the two cotyledons that are positioned opposite each other at the top of the hypocotyl (Figures 1B and 1C). The SAM is connected by vascular tissue to the vascular bundle of the hypocotyl at the point where the vascular bundles branch into the petioles of the cotyledons (Figure 1D). A few days after germination, the SAM produces the first true leaves (Figures 1B and 1D).

In most cases, *nam*⁻ seedlings fail to produce the first leaves (Figure 1A), and the seedling is thereby arrested in growth and dies after a few weeks. The petioles of the cotyledons of *nam*⁻ seedlings are fused up to the base of the cotyledon blade. Furthermore, the relative position of the cotyledons is variable in different *nam*⁻ seedlings (Figure 1E). In seedlings in which the angle between the cotyledons is small, the cotyledons are often laterally fused. In the most extreme cases, this leads to the formation of a single cotyledon disc. Scanning electron microscopy (SEM) and serial sectioning shows that most *nam*⁻ seedlings completely lack the typical SAM cells but instead contain a group of large highly vacuolated cells at the site normally occupied by the SAM (Figures 1F, 1G, and 1H). These large cells do not resemble any of the surrounding cells.

To determine whether the absence of a SAM resulted from a defect during embryogenesis, we examined serial sections through torpedo stage and mature wild-type and *nam*⁻ embryos. At the late torpedo stage, all embryos derived by selfing of a wild-type plant contained typical intensely stained cells at the epiphysis, the position at which the SAM is formed (27 embryos analyzed, Figure 2A). In examining the development of 46 embryos obtained by self-pollination of *NAM*⁺/*nam*⁻ plants, we found 12 embryos that lacked these intensely stained cells (Figure 2B). Similarly, serial sectioning showed that 8 of 37 mature embryos from *NAM*⁺/*nam*⁻ plants lacked typical SAM cells at the epiphysis (compare Figures 2C and 2D). At the position of the SAM, these embryos contained cells that resembled cotyledon–petiole cells. Apparently, the large vacuolated cells seen in *nam*⁻ seedlings appear after germination.

A fraction of *nam*⁻ seedlings eventually develop a shoot (to which we will refer as an "escape shoot"), indicating that they contain a meristem (see Figure 1I). The percentage of *nam*⁻ seedlings developing escape shoots is highly variable, even in progeny derived from different seed capsules of the same parental plant. In Figure 1J, a *nam*⁻ seedling is shown that develops a normal-looking SAM at the same position as in wild-type seedlings (i.e., just above the point where the petioles of the two cotyledons branch). This meristem is surrounded by the same large cells found in *nam*⁻ seedlings without a SAM (see Figure 1K). Some escape shoots develop into mature plants, whereas others only develop a few leaves that remain small and then cease to grow any further. Serial sectioning showed that the vascular system in escape shoots was not always connected to that of the hypocotyl and root (e.g., the escape shoot shown in Figure 1L), which may be a reason why some escape shoots are arrested in growth.

These data indicate that the *nam* gene product is required during embryogenesis for the establishment of the SAM and correct positioning of the cotyledons.

The *nam* Gene Product Is Required for Normal Flower Development

The plants that develop from escape shoots have normal-looking stems, leaves, and axillary shoots, but the

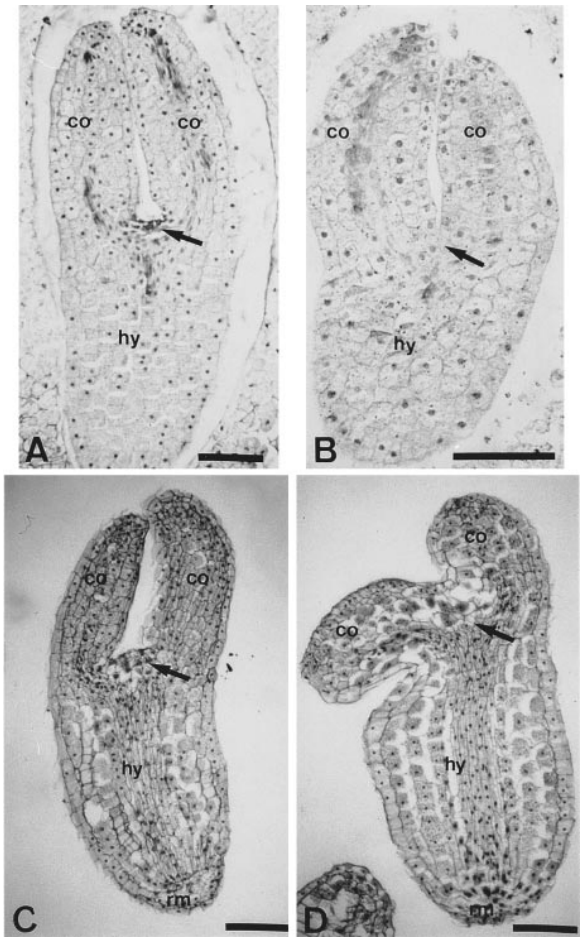

Figure 2. Phenotypic Comparison of Wild-Type and *nam*⁻ Embryos

Wild-type plants and plants heterozygous for the *nam-S227* allele were self-pollinated, and embryos were serially sectioned.

(A) Wild-type torpedo-stage embryo containing densely stained SAM cells.

(B) *nam*⁻ torpedo-stage embryo. No SAM cells are developed between the cotyledons.

(C) Wild-type mature embryo.

(D) *nam*⁻ mature embryo.

(The position of the SAM is indicated by an arrow. co, cotyledon; hy, hypocotyl; rm, root meristem. The scale bar indicates 100 μm.)

flowers show various abnormalities. In all flowers, the number of petals is increased, as calculated by the number of central veins. This causes the limb of the corolla to become crumpled (Figure 3B). Anthers are always fused to the extra petals (Figures 3B and 3J). In most (but not all) flowers, small organs, which consist of antheroid tissue with stigmatic tissue on top, develop between the whorl of stamens and the carpels (Figure 3C). The phenotype of the central gynoecium is variable even between different flowers on the same plant. In some flowers, it has an almost normal appearance (Figure 3C), whereas in other flowers, one of the carpels does not develop properly, leading to a deformed and bent gynoecium structure (Figures 3D and 3J). In the most extreme cases, antheroid tissue develops at the base of the style (Figure 3D). Inside the ovary, the placenta and ovules develop aberrantly. The tip of the placenta is often not properly attached to the carpels and carries a reduced number of ovules. As a consequence of these floral abnormalities, plants originating from an escape shoot are male- and female-sterile.

To analyze the abnormalities seen in mature *nam*⁻ flowers more precisely, we examined young flower buds by SEM. In wild-type Petunia flower buds, primordia of whorls 1, 2, and 3 develop into five sepals, five petals, and five stamens, respectively (Figure 4A). In whorl 4, the center of the bud, two carpel primordia emerge that will later fuse to form one gynoecium. Differences between wild-type and *nam*⁻ flowers can be seen as soon as the first petal primordia in whorl 2 emerge (Figure 4B). In wild-type flower buds, these petal primordia are positioned alternating with the primordia in whorls 1 and 3 (Figure 4A). In young *nam*⁻ flower buds, additional whorl 2 primordia (indicated with an asterisk in Figures 4B and 4C) emerge between the primordia in whorls 1 and 3. At a later stage of development, another five primordia emerge in whorl 2 in the normal position (Figure 4C). The occurrence of ten primordia in whorl 2 leads to the formation of heart-shaped petal structures originating from laterally fused petal primordia (Figure 4D). Later in development, the extra petals in whorl 2 fuse to the stamens developing in whorl 3. Note that in the floral bud shown in Figure 4C, a small extra primordium emerges between whorl 3 and whorl 4 that presumably would have developed into one of the small antheroid organs often seen in *nam*⁻ flowers (see Figure 3C). Because the aberrations in whorl 4 organs are variable between different flowers, we did not attempt to analyze these defects further by SEM.

In *clavata1*⁻ flowers of Arabidopsis, creation of extra space by enlargement of the floral meristem correlates with, and possibly causes, the development of extra organs (Clark et al., 1993). However, the diameter of wild-type and *nam*⁻ floral meristems, as measured by SEM, does not differ significantly, indicating that *nam* does not affect floral meristem size.

Interaction of *nam* with a Homeotic Floral Organ Identity Gene

To investigate whether the doubling of primordia seen in the second whorl of *nam* mutant flowers is petal specific or position specific, we constructed double mutants of the *nam-S227* allele and the *green petal-R100* allele. The *green petal* gene (*gp*) is homologous to the floral organ identity gene *deficiens* of Antirrhinum and encodes a MADS domain type of transcription factor (Kush et al., 1993; van der Krol et al., 1993). In *gp*⁻ plants, petals are replaced by sepals, whereas organs in other whorls seem to be unaltered (see Figure 3H). We crossed the *gp* mutant to a plant heterozygous for the mutant *nam-S227* allele and selected the double mutant from F2 *nam*⁻ seedlings that developed an escape shoot. In flowers of this double mutant (see Figure 3F), each second-whorl organ consisted of two laterally fused sepals, indicating that extra second-whorl organs were formed (see Figure 3G). The third- and fourth-whorl organs were very deformed. They consisted of a ring of antheroid and carpeloid structures around a “naked” placenta with ovules (see Figures 3H and 3K), while

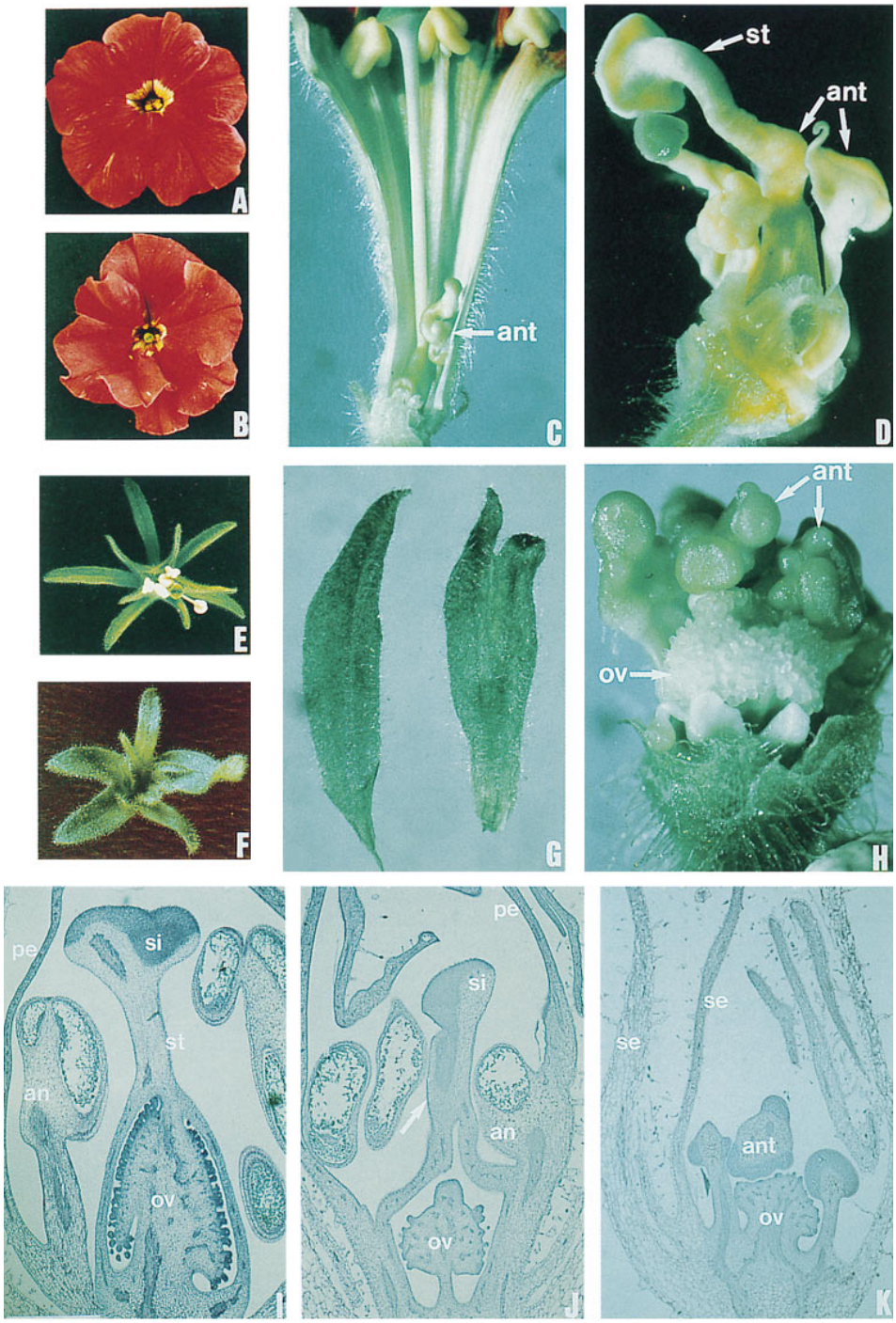

Figure 3. Mutant Phenotypes of *nam-S227* Flowers and *nam-S227/gp-R100* Double-Mutant Flowers

(A) Wild-type Petunia flower.

(B) *nam*$^{-}$ flower on an escape shoot. Extra petals are formed in whorl 2, leading to a fusion of the anthers to these extra petals.

(C) Opened *nam*$^{-}$ flower. Note the extra antheroid structures (ant) irregularly formed in *nam*$^{-}$ flowers.

(D) Aberrant gynoecium structure in *nam*$^{-}$ flowers. The outer three whorls of the flower are removed. Two extra carpeloid/antheroid structures aside the aberrant central gynoecium with antheroid tissue are visible at the lower part of the style.

(E) *gp*$^{-}$ flower.

(F) Flower on an escape shoot of a *nam/gp* double mutant.

(G) Second whorl organ from *gp*$^{-}$ flower (left) and *nam*$^{-}$/*gp*$^{-}$ flower (right).

(H) Inner whorl organs found in *nam*$^{-}$/*gp*$^{-}$ flowers. The two outer whorls are removed, as well as whorl 3 tissue in the front.

(I–K) Microscopical sections through (I) wild-type, (J) *nam*$^{-}$, and (K) *nam*$^{-}$/*gp*$^{-}$ flower. In (J), an incompletely developed carpel is indicated by an arrow. The scale bar in (I) indicates 1 mm; (J) and (K) are shown at the same magnification.

(ant, antheroid structures; st, style; si, stigma; ov, ovary; an, anther; pe, petal; se, sepal.)

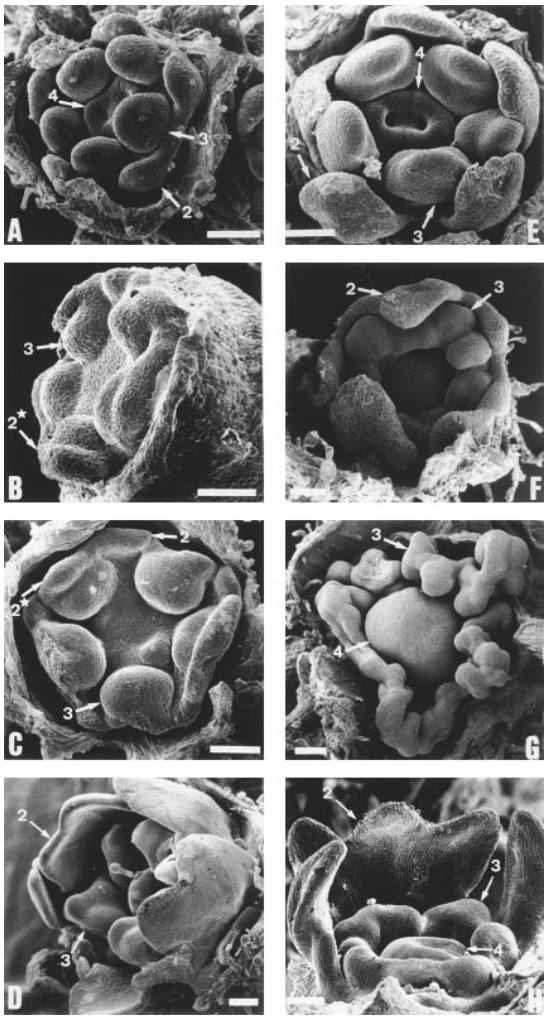

Figure 4. Developmental Changes in Young *nam-S227* Flowers and *nam-S227/gp-R100* Flowers

Scanning electron microscopy was performed on wild-type, *nam*$^-$, *gp*$^-$, and *nam*$^-$/*gp*$^-$ flower buds.
(A) Wild-type floral meristem.
(B) *nam*$^-$ floral meristem. Extra petal primordia indicated by 2* are formed in whorl 2, opposite the stamen primordia.
(C) Top view of *nam*$^-$ floral meristem.
(D) *nam*$^-$ floral meristem at a later stage in development. The second whorl organs consist of two fused petal primordia.
(E) *gp*$^-$ floral meristem.
(F) *nam*$^-$/*gp*$^-$ floral meristem. The third whorl seems to consist of a ring of numerous fused primordia.
(G) Top view of *nam*$^-$/*gp*$^-$ floral meristem of slightly further developed bud.
(H) *nam*$^-$/*gp*$^-$ floral meristem at a later stage in development.
First-whorl organs were removed in all cases. In (G), also, second-whorl organs were removed. The scale bar equals 100 μM in all panels. Whorl numbers are indicated with arrows.

other gynoecium structures such as the ovary wall, the style, and stigma were completely missing.

SEM analyses of young *gp*$^-$ flowers showed that the number and position of organ primordia were basically identical to those of wild-type flowers (Figure 4E). As in flowers of *nam*$^-$ mutant plants, *nam*$^-$/*gp*$^-$ double mutant flowers had ten primordia in whorl 2 (Figure 4F). In whorl 3, a ring of an undefined number of primordia emerged at the place where, normally, five stamen primordia develop (Figures 4F and 4G). Furthermore, the two carpel primordia in whorl 4 were not formed. At later developmental stages, the domelike structure in the center of the flower rose to form the placenta with ovules (Figure 4G).

The observation that the number of primordia in whorl 2 was equally increased in flowers of *nam*$^-$ or *nam*$^-$/*gp*$^-$ plants indicates that NAM does not act petal specific. In *nam*$^-$/*gp*$^-$ plants, however, an increased number of third-whorl organ primordia was also formed, while normal fourth-whorl carpel primordia were missing.

Molecular Cloning of the *nam* Gene

All analyzed transposon-induced mutants isolated in the Petunia W138 background were due to insertions of *dTph1* transposable elements. Therefore, we expected the *nam-S227* allele also to harbor a *dTph1* insertion. We cloned nine distinct *dTph1* flanking sequences that were unique for a family of *nam-S227* plants (Souer et al., 1995), one of which (clone *es87*) fully linked to the *nam* locus in the 18 *NAM*$^+$/*nam*$^-$ and *NAM*$^+$/*NAM*$^+$ plants tested (data not shown). We partially sequenced a genomic clone isolated from a *NAM*$^+$/*NAM*$^+$ line that corresponded (by hybridization and sequence) to *es87*.

To prove that the cloned genomic fragment indeed contained the *nam* gene, additional transposition events at the locus needed to be analyzed. Because *nam*$^-$ plants are sterile, we could not obtain revertant progeny originating from germinal transposon excisions. We also could not detect any somatic revertant sectors or branches on plants homozygous for the *nam-S227* allele. As an alternative, we screened 2000 Petunia W138 plants for new *dTph1* insertions in the cloned genomic region by a polymerase chain reaction (PCR)-based assay (Koes et al., 1995) and identified three (wild-type) plants that were heterozygous for a wild-type allele and a *dTph1* insertion allele (*nam-V2009*, *nam-V2208*, and *nam-V2213*). The exact insertion sites were determined by PCR and sequence analysis (Figure 5B). Selfing of each of these heterozygous plants yielded progeny segregating 3:1 for wild-type and the *nam*$^-$ phenotype (Table 1, crosses 2–4). To confirm that the new mutations were allelic, we crossed heterozygous plants harboring the four different insertion alleles in various combinations. In all crosses, we found about 25% progeny seedlings with the *nam*$^-$ phenotype (Table 1, crosses 5–10). For all (combinations of) *nam* alleles, we found that *nam*$^-$ seedlings produced occasional escape shoots that developed into mature plants with the same abnormalities in their flowers as described for the original *nam-S227* allele.

One plant originating from an escape shoot on a *nam-V2009/nam-V2009* seedling produced, after formation of several branches with mutant flowers, a revertant branch bearing wild-type flowers. Cross-pollination of such flowers yielded a progeny segregating for wild-type and *nam*$^-$ seedlings (Table 1, cross 11). PCR analysis showed that wild-type plantlets contained a revertant allele that had lost the *dTph1* insertion, indicating that the phenotypic reversion in the parental plant was due

(B)

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GATCTTAGGTTTAAAATTTAGCACTATTAGATCATTTCTTTCTATTTGTCCAAATTTTTTTAAATGGAATTACTC
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                                  M  E  N  Y  Q  H  F  D  C  S  N  S  N  L  P  P
TTTCCTTTGCTATAATTCTTGTTTTCAAATGGAGAACTACCAGCATTTCGACTGCAGTGATTCAAATCTTCCCCC
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TGGCTTTAGGTTCCATCCAACTGATGAAGAGCTCATCACTTACTACTTACTAAAGAAAGTTCTTGACAGCAACTT
                                                  S227
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                                                              E  K  A  K  M
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                                                                  D  E
TATTACTTTCTGTTACATCTATTACGCAATGTCATGATGAGCTAAGTAATATTTATGATTTTCTTTTCAGGACGA
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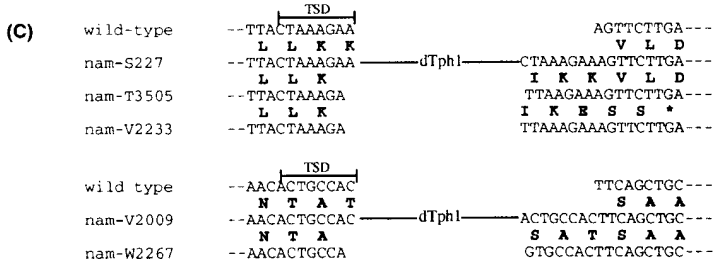

Figure 5. Sequence and Structure of the *nam* Gene and Mutant Alleles

(A) Genomic map of the *nam* gene. Thick bars represent exon sequences. The position of *dTph1* insertions is indicated by open triangles. Primers used are indicated below the map.

(B) Nucleotide and deduced amino acid sequence of the *nam* gene. The target site duplication sequence of *dTph1* insertion alleles is indicated by bracketed lines beneath the sequence. The end of the longest mRNA 5′ RACE product is underlined.

(C) Footprint alleles generated after excision of *dTph1* from the insertion alleles *nam-S227* and *nam-V2009*. The 8 bp duplicated upon dTph1 insertion are marked TSD. Plants carrying the footprints originating from *nam-S227* are mutant, while the footprint from *nam-V2009* leads to a reversion to wild-type.

Table 1. Independently Isolated *nam* Mutants Are Allelic

Cross	Genotype	Wild-type	Mutant		Total
			nam	*nam* with escape shoot[b]	
1	*NAM*$^+$/nam-S227 × *NAM*$^+$/nam-S227	61	20	3 (13%)	84
2	*NAM*$^+$/nam-V2009 × *NAM*$^+$/nam-V2009	188	46	15 (25%)	249
3	*NAM*$^+$/nam-V2208 × *NAM*$^+$/nam-V2208	348	87	18 (17%)	453
4	*NAM*$^+$/nam-V2213 × *NAM*$^+$/nam-V2213	288	84	9 (10%)	381
5	*NAM*$^+$/nam-S227 × *NAM*$^+$/nam-V2009	68	25	8 (24%)	101
6	*NAM*$^+$/nam-S227 × *NAM*$^+$/nam-V2208	631	183	12 (6%)	826
7	*NAM*$^+$/nam-S227 × *NAM*$^+$/nam-V2213	316	91	2 (2%)	409
8	*NAM*$^+$/nam-V2009 × *NAM*$^+$/nam-V2208	396	67	39 (37%)	502
9	*NAM*$^+$/nam-V2009 × *NAM*$^+$/nam-V2213	336	67	65 (49%)	468
10	*NAM*$^+$/nam-V2208 × *NAM*$^+$/nam-V2213	154	38	3 (7%)	195
11	*nam*-V2009/*nam*$^+$-V2267 × *nam*-V2009/*nam*$^+$-V2267[a]	54	6	1 (14%)	61
12	*NAM*$^+$/nam-T3505 × *NAM*$^+$/nam-T3505	113	19	5 (21%)	138

[a] Revertant branch from *nam*-V2009/*nam*-V2009 plant.
[b] Percentage of *nam* seedlings with escape shoot is indicated between brackets.

to the excision of *dTph1* in the L2 tunica layer. Partial sequencing of the revertant allele from one homozygous wild-type plant showed that the *dTph1* excision had generated a 6 bp footprint that leaves the open reading frame intact (Figure 5C).

Taken together, these data show that transposon insertions and excisions in the cloned genomic fragment fully correlate with phenotypic changes. Thus, this fragment contains part of the *nam* gene, and both the seedling and the flower phenotype are caused by mutation of this single gene.

Structure of the *nam* Gene

To determine the structure of the *nam* gene, we attempted to isolate *nam* cDNA clones. Screens of various cDNA libraries were unsuccessful, presumably owing to the very low *nam* mRNA levels. However, the homology of the *nam* gene with several Arabidopsis and rice cDNA clones (see below) enabled us to identify, in the genomic sequence, a putative translation start codon 93 bp upstream of the *dTph1* insertion site in *nam-S227*. Using primer nam4, complementary to the putative translation start, and an oligo(dT) primer, we could amplify an 1174 bp cDNA fragment from reverse-transcribed mRNA isolated from young carpels. This fragment contains a 981 bp open reading frame specifying a 327 amino acid protein, as shown in Figure 5B. The upstream 85 bp of mRNA sequence amplified by 5′ rapid amplification of cDNA ends (RACE)–PCR contained stop codons in all frames, confirming that we had correctly inferred the translation start (Figure 5B). Based on the comparison of the genomic and cDNA sequence, the *nam* gene contains two introns of 302 and 627 bp, respectively.

Homology of *nam* to Other Genes

Using the PROSITE and BLOCKS directories (Bairoch, 1992; Henikoff and Henikoff, 1991), we could not detect any of the known patterns in the predicted NAM protein sequence except for some common features, such as myristoylation and phosphorylation sites. When we used the deduced NAM protein sequence as a query for the SWISSPROT database, no clear homologies were detected. However, when we used the *nam* DNA sequence as a query for the EMBL sequence database, we found strong homology to several Arabidopsis and rice cDNA clones in the region encoding the N-terminal part of the NAM protein (Figure 6). Most of these sequences were derived from randomly isolated cDNA clones (expressed sequence tags) except for two Arabidopsis clones, ATAF1 and ATAF2, isolated by Hirt et al. (personal communication; see Discussion). No significant matches were found in databases containing sequences from yeast, Caenorhabditis elegans, and human expressed sequence tags and genomic regions, despite the large number of genes covered.

Further examination of the NAM protein reveals some striking features. The conserved N-terminal part of the NAM protein consists of many charged residues. The first 150 amino acids contain 20% basic residues (R, K, and H) and 13.4% acidic residues (D and E). The C-terminal part after the first 150 amino acids of the NAM protein contains relatively high percentages of serine (23.9%), proline (8.0%), and leucine (8.0%) residues. The same holds for the proteins encoded by Arabidopsis and rice cDNA clones, suggesting that this may be of functional significance.

Stable Recessive *nam* Alleles Still Allow Escape Shoots to Be Produced

For all four *dTph1* insertion alleles, *nam*$^-$ seedlings were found that occasionally produced escape shoots. To find out whether this was due to the inherently unstable character of *dTph1* insertion alleles, we searched for stable recessive *nam*$^-$ alleles. To this end, we analyzed various plant families obtained from selfings of *NAM*$^+$/*nam-S227* heterozygous plants by PCR and Southern blotting. We found one family of plants, T3505, consisting of approximately 25% *nam*$^-$ seedlings, some of which produced escape shoots (Table 1, cross 12), even though we could not detect a *dTph1* element at the *nam* locus in either wild-type plants or escape shoots. Selfings of *NAM*$^+$/*nam*$^-$T3505 plants again segregated 3:1 for *nam*$^-$ seedlings that still produced occasional escape shoots. From one escape shoot, we amplified the region around the original *dTph1* insertion and sequenced the excision site (see Figure 5C). In the *nam-T3505* allele, the footprint left behind after transposon

Figure 6. Alignment of the Deduced Amino Acid Sequences of *nam* and cDNAs from Arabidopsis

The diagram shows only the comparison of *nam* with the two Arabidopsis cDNA clones ATAF1 and ATAF2, as isolated by Hirt et al. (GenBank accession numbers X74756 and X74755, respectively). Amino acids identical in all three of the sequences are shown in closed boxes. Another nine partial sequences of expressed sequence tags from Arabidopsis (GenBank accession numbers T21197, T44589, T76783, T76864, T41582, T45210, T46511, R30004, R90134, and R89957; Höfte et al., 1993; Newman et al., 1994) and rice (GenBank accession numbers D21968, D21966, D21959, D22845, and D15387; Sasaki et al., 1994; M. Yuzo and S. Takuji, unpublished data) share the same conserved domains but are not shown in the diagram.

excision added 6 bp to the open reading frame, thereby changing the amino acid sequence from LLKKVL to LLK*IK*KVL (see Figure 5C), apparently abolishing normal NAM protein function.

A second stable recessive allele, *nam-V2233*, was found in the F2 family from which we selected the *nam/gp* double mutants. PCR analysis showed that one plant developing from an escape shoot completely lacked the *dTph1* element in both alleles of the *nam* gene. In *nam-V2233*, excision of *dTph1* from the *nam-S227* allele had introduced a frame shift (see Figure 5C). The NAM reading frame now ends with a stop codon a few triplets after the excision site, in a region encoding the conserved N-terminal part of the NAM protein.

Based on these results, we conclude that the formation of escape shoots can still occur in the complete absence of a functional NAM protein.

Expression Pattern of the *nam* Gene

To study the mechanism by which *nam* contributes to pattern formation, we analyzed its expression by in situ hybridization. We specifically focused on developing embryos and flower buds, since the mutant phenotype indicated that these were sites where *nam* should be active.

During embryogenesis, *nam* transcripts first became detectable upon transition from heart to torpedo stage in a few cells that surround the developing SAM (Figure 7A). In torpedo-stage embryos, *nam* expression had become much stronger and was limited to two stripes of cells around the developing SAM (Figure 7B). Examination of serial sections showed that these two stripes were in fact part of a ring around the developing SAM.

In the inflorescence meristem, *nam*-expressing cells were first detected as two stripes (Figure 7C). Sections through an apex at a slightly later developmental stage showed that these stripes marked the boundaries where bracts were formed. Furthermore, a third stripe of *nam*-expressing cells was now apparent at the boundary between the young floral meristem and the inflorescence meristem (Figure 7D).

Once the floral meristem had generated sepals in whorl 1, and primordia in whorls 2 and 3 were just recognizable, *nam* expression was detected around the stamen primordia (Figure 7E). In sections through the center of a stamen primordium, *nam*-expressing cells appeared to form two stripes, while in sections that just missed the primordium, *nam*-expressing cells formed a sector (the stamen primordia on the left and right, respectively, in Figure 7E). Thus, *nam* was expressed in rings around the stamen primordia. The same expression pattern was observed in younger floral buds in which whorls 2 and 3 primordia were not yet visible, indicating that *nam* expression precedes primordium formation (data not shown).

At a slightly later stage, *nam* expression is detectable between the two carpel primordia and in the developing anthers at the site where sporogenic tissue will be formed (Figure 7F). Once the placenta starts to develop in the center of the bud, *nam* becomes expressed at the tip of the placenta, the site that will later fuse to the carpel wall (Figure 7G). By the time the two carpels and the placenta start to fuse, ovule development initiates (Angenent et al., 1995b). At this stage, *nam* is expressed in an apparently striped pattern along the placenta edge, presumably marking the boundaries of ovule primordia (Figure 7H). During ovule development, stripes of *nam*-expressing cells become evident within the ovule that again seem to form a ring. This expression pattern presumably marks the sites at which, slightly later, the integuments will form (Figure 7I).

Discussion

By mutational approaches, a series of genes has been isolated in recent years that performs key functions in pattern formation during plant development (Haughn et al., 1995; Jürgens, 1995; Weigel and Meyerowitz, 1994). The large majority of genes analyzed so far are expressed inside the young developing primordia or meristems affected by the mutation and play a role in establishing or maintaining their identity. The *nam* gene described in this paper has a very different expression pattern: *nam* is expressed at the boundaries of a variety

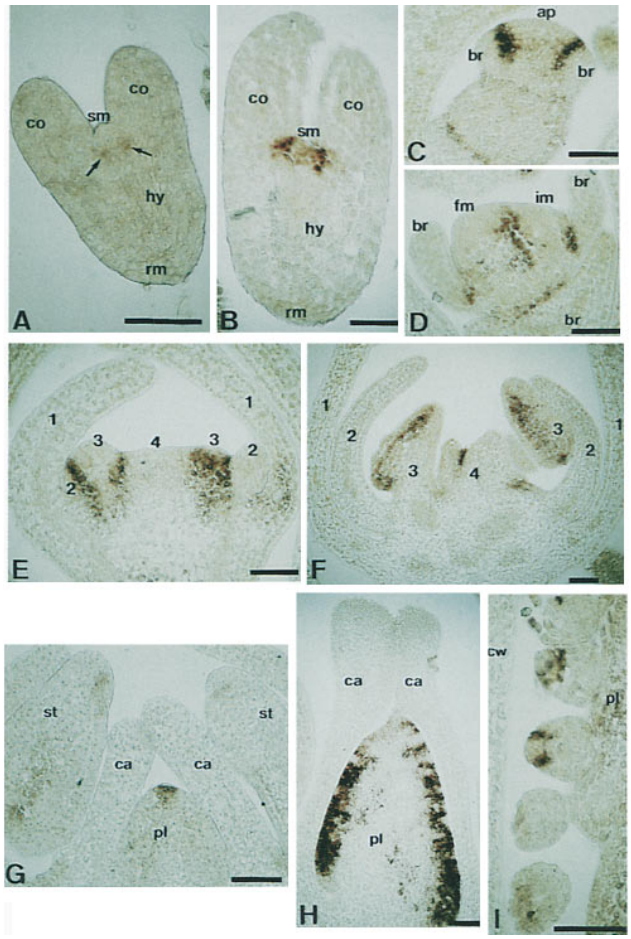

Figure 7. In Situ Localization of *nam* mRNA in Wild-Type Petunia Embryos and Inflorescences

(A) Late heart-stage embryo. Arrows point to the cells that are at this stage weakly expressing *nam*.

(B) Torpedo-stage embryo. *nam* expression is limited to a ring around the developing SAM.

(C) Inflorescence apex. The two vertical stripes of *nam*-expressing cells are at the boundaries of the site at which bract primordia will appear. The horizontal *nam* stripe at the bottom marks the boundary of a bract formed at an earlier node.

(D) Inflorescence apex with two bracts. Note that *nam* expression separates the two bracts and the floral and inflorescence meristems.

(E) Young flower bud that has just developed petal and stamen primordia. The numbers indicate the position of the four whorls; primordia in whorl 4 are not yet visible at this developmental stage. On the left side of the flower, this section is through the middle of a whorl 3 primordium and between the whorl 2 promordia. On the right side of the flower, the section is between whorl 3 primordia and through the middle of a whorl 2 primodium.

(F) Flower bud in which whorl 4 primordia have just appeared. Expression is restricted to the sporogenous cells in the stamen, the boundary between carpel and stamen, and between the developing carpel primordia.

(G) Carpels and very young placenta. Expression is restricted to the sporogenous cells of the stamen, the boundary between stamen and carpel, and the top of the developing placenta.

(H) Carpels later in development. The placenta is connected to the carpels, but ovule primordia are not yet evident. Note the striped appearance of *nam* expression along the edge of the placenta.

(I) Young ovules before integument (primordia) are formed.

All tissues were sectioned longitudinally and hybridized with the anti-sense strand spanning the (gene-specific) 3′ half of the *nam* cDNA. Control section hybridized with the sense RNA strand from the same fragment did not produce a signal (data not shown). Scale bars indicate 100 μm.

(sm, shoot meristem; co, cotyledon; hy, hypocotyl; rm, root meristem; br, bract; ap, apex; fm, floral meristem; im, inflorescence meristem; st, stamen; ca, carpel; pl, placenta; cw, carpel wall. Numbers indicate whorls.)

of primordia and meristems. We propose that the major role of *nam* is specification of the position, rather than the identity, of primordia and meristems.

nam Is a Member of a Small Gene Family Encoding a Novel Class of Proteins

We have isolated the *nam* gene as a sequence flanking the Petunia transposable element *dTph1*. NAM belongs to a novel class of proteins that all share a common N-terminal domain of about 150 amino acids that is conserved in both dicot (Arabidopsis) and monocot (rice) plants, but that may be absent in yeast and animal genomes. Two of the Arabidopsis cDNA clones homologous to *nam* (ATAF1 and ATAF2) were isolated by their capability to activate a cauliflower mosaic virus 35S promoter construct in yeast (H. Hirt, personal communication). This suggests some role for NAM homologous proteins in transcriptional control.

The Petunia genome contains at least four additional *nam* homologous genes, which each contain the conserved N-terminal domain (unpublished data). It is possible that these homologs are also involved in pattern formation. The observation that phenotypic alterations are seen only in a subdomain of the *nam* expression pattern indicates that some *nam*-homologs may be (partially) redundant with *nam* itself.

Role of *nam* in SAM Formation During Embryogenesis

In Arabidopsis, mutants have been identified in which the apical–basal pattern of a plant embryo is altered. Usually, one of the basic body parts is missing (reviewed by Goldberg et al., 1994; Jürgens, 1995; Jürgens et al., 1994). The *nam* mutant of Petunia lacks a SAM, the most terminal element along the apical–basal axis, and thus belongs to this category of mutants.

The mutants *amputata* of Antirrhinum and *zwille* and *stm* of Arabidopisis resemble *nam*$^{-}$ seedlings in that they also fail to produce a SAM (Jürgens et al., 1994).

For *stm* mutants only, the defect was traced back to an aberration during embryogenesis (Barton and Poethig, 1993; Long et al., 1996). The *stm* gene encodes a homeodomain protein and is expressed in SAM (precursor) cells from late globular stage onward. Because *stm* expression continues into the mature plant, it is thought to maintain meristem function by keeping the cells in an undifferentiated state. As *nam* encodes a different type of protein and displays a completely different expression pattern, it must affect meristem formation by a different mechanism. In torpedo-stage and mature *nam*$^-$ embryos, cells that resemble cotyledon–petiole cells are found at the site normally occupied by SAM cells. One possibility is that the normal SAM (precursor) cells adopt a different fate in the mutant, while cell lineage remains unaltered. Considering that *nam* is expressed in cells that surround the SAM, this would imply that *nam* acts cellnonautonomously, possibly by mediating in intercellular signaling. Another possibility is that development of SAM cells is inhibited because their space is occupied by cells from the surrounding tissue. By analogy with the role of *nam* in flower development (see below), it is conceivable that *nam* expression interferes with cell division around the developing SAM in the embryo. In the absence of functional NAM protein, proper development of the SAM may be hampered because of the proliferation of neighboring cells. The observation that the Arabidopsis *stm* gene (Long et al., 1996) was activated much earlier than *nam* during SAM development is consistent with the latter scenario, but difficult to fit into the first. Analyses of cell lineages and of expression of the Petunia *stm* homolog may help to define further the role of *nam* during embryogenesis.

Occasionally, *nam*$^-$ seedlings contain some meristimatic cells in the normal SAM position, which can give rise to an escape shoot. The occurrence of this meristem is unpredictable and not well understood. It seems unlikely that escape shoots result from residual *nam* activity; all isolated *nam* alleles (including the stable recessive *nam-T3505* allele) have indistinguishable phenotypes, indicating that they are all null alleles. Because *nam* is a member of a gene family, escape shoots may result from partial redundancy of *nam* function, similar to the proposed mechanism by which *squamosa* mutants of Antirrhinum produce occasional escape flowers (Huijser et al., 1992). In a scenario in which SAM formation in *nam* mutants is repressed by proliferation of neighboring cells, it is conceivable that some SAM cells escape repression and organize themselves at a later stage into a functional meristem. This may explain why escape shoots generally appear much later than wild-type shoots and are not always properly connected to the vascular system of the hypocotyl and root.

Role of *nam* in Postembryonic Development

Genes that determine the pattern of the embryo may also be involved in pattern formation at later developmental stages. The *emb*/*gnom* and *stm* genes of Arabidopsis (Long et al., 1996; Shevell et al., 1994) and *nam* are expressed in adult plant tissues, suggesting that this may be the rule rather than the exception. Because most mutants with an altered seedling pattern do not develop much further than the seedling stage, the role of the corresponding genes during postembryonic development, if any, cannot be inferred from a mutant phenotype. The occurrence of occasional escape shoots enabled us to study the role of NAM in postembryonic development, also at the phenotypic level.

Comparison of the phenotype of *nam*$^-$ mutants with the expression pattern indicates that phenotypic alterations are seen only in a subdomain of the *nam* expression pattern. Low amounts of *nam* mRNA are detectable in stem and young leaves (unpublished data), but no phenotypic alterations could be detected in leaves, stems, axillary meristems, or other vegetative structure on *nam* escape shoots. Similarly, *nam* mRNA is expressed at several sites in the inflorescence apex (Figures 7C and 7D), but no corresponding phenotypic changes are observed in *nam*$^-$ inflorescences. One possibility is that the detected *nam* mRNA is not expressed (e.g., because of posttranscriptional regulation mechanisms). Considering that *nam* is a member of a gene family, it seems more likely that *nam* function is redundant on these sites.

The most striking feature of the *nam* expression pattern in the inflorescence is that it marks the boundaries between different primordia before the separation between primordia and meristems has become visibly evident (Figures 7A–7H). The phenotype of *nam*$^-$ flowers provides evidence that *nam* is required here to prevent these regions from developing into a primordium (at least in the cells around the stamen primordia in whorl 3). In *nam*$^-$ flowers, the whorl 2 cells on the outside of stamen primordia are no longer prevented from developing into a primordium. Similarly, on the inside of stamen primordia, *nam*$^-$ flowers occasionally contain extra organ primordia (Figures 3C, 3D, and 4C), and this effect is dramatically enhanced in a *gp*$^-$ background (Figures 3E–3H and 4E–4H). In *nam* mutants, the extra primordia in whorl 2 develop into petals in a *GP*$^+$ background and sepals in a *gp*$^-$ background, suggesting that floral organ identity and position are separate processes. However, the increase in primordium formation in whorl 3 of *nam*$^-$/*gp*$^-$ flowers suggests that either *gp* or the identity of whorl 2 organs is somehow involved in the regulation of primordia formation in whorl 3. The failure of *gp*$^-$/*nam*$^-$ flowers to initiate proper carpel primordia in whorl 4 may be a secondary effect caused by the proliferation of primordia in whorl 3.

To understand how NAM prevents primordium formation, it is important to consider how a meristem with a smooth surface converts into a structure with bulges (primordia) located in specific sites. Quantitative analysis of surface expansion behavior of *Anagallis* floral meristems leads to the conclusion that cell division and cell expansion in primordial regions differs in rate and direction from that in the regions between (Hernández et al., 1991). This conclusion is supported by cell lineage analysis in Antirrhinum flowers, using a temperature-sensitive transposon (Vincent et al., 1995). This study reveals that at early stages of development and before organ identity becomes apparent, lineages are not restricted between petals, stamens, and carpels. Shortly after, restrictions arise between whorls, and this correlates with the onset of expression of genes that control

organ identity. This can be explained by assuming that the boundary regions between whorls adopt particular growth rates, or that they adopt orientations that reduce the chance of cells contributing descendants to more than one whorl, or both. Based on this, *nam* may influence the rate or the direction, or both, of cell division and expansion in the regions between primordia. A similar function has recently been proposed for the *superman* (*sup*) gene of Arabidopsis (Sakai et al., 1995). Mutant *sup* flowers develop extra stamens interior to the normal third-whorl stamens. In situ hybridization has revealed early *sup* expression adjacent to the boundary of the third and fourth whorls and, at later stages, in the inner part of whorl 3. Both the phenotypic and molecular data could be explained by assuming a role for SUP in cell division control at the boundary of stamen primordia.

Hernández and Green (1993) have proposed that primordia may initiate as bumps that are pushed out of the meristem by mechanical forces. In such a model, *nam* could act to create sites of low flexibility that locally suppress the formation of primordia.

Experimental Procedures

Plant Material

In a random transposon–mutagenesis experiment (unpublished data), we visually screened about 2000 F2 families originating from 50 different parental plants of the Petunia line W138 and found one family that segregated for the *nam*$^-$ phenotype. The EMS-induced *gp*-R100 allele used for double-mutant analysis appears to be a null allele, since it is equally as strong as the X-ray–induced complete deletion allele of the same gene (Angenent et al., 1995a).

Isolation and Sequence Analysis of Genomic and cDNA Clones

The es87 clone contained an IPCR-amplified *dTph1* flanking sequence that genetically linked to *nam* (Souer et al., 1995). For the isolation of genomic clones, a library of the Petunia line W137 was screened with es87 insert. Two hybridizing plaques were purified to homogeneity, one of which remained hybridized to es87 after stringent washing (0.1× SSC/0.1% SDS, 65°C). Fragments of this genomic clone were subcloned into pBluescript-KS plasmids (Stratagene) and sequenced. The sequence of es87 and the genomic clone matched exactly, confirming that both derived from the same locus.

The *nam* cDNA was amplified from total RNA isolated from carpels, stage 2, as follows: 2.5 μg total RNA was boiled in 11.5 μl sterile water for 1 min and immediately cooled on ice. Then, 10 μl 5× RT buffer, 5 μl 100 mM DTT, 20 μl dNTPs (2.5 mM each), 2.5 μl oligo(dT)–RACE primer 5′-dGACTCGAGTCGACATCGA(T)17-3′ (10 pmol/μl), and 1.0 μl M-MLV RT (200U/μl) were added. Reactions were incubated for 1 hr at 42°C. Part of this reaction (5 μl) was PCR amplified, using the primers nam4 (5′-dCGGGATCCCATGGAGAAC TACCAGCATTTC-3′) and the RACE primer (5′-dGACTCGAGTCGAC ATCG-3′). PCR products were cloned in BamHI–SalI-digested pBluescript-KS and sequenced.

For amplification of the 5′ end of the *nam* cDNA, polyA$^+$ mRNA was isolated from young floral buds (<1 cm), using an Oligotex mRNA kit (Qiagen), and cDNA was prepared as described above. The 5′ end of the cDNA was amplified, using an AmpliFINDER 5′ RACE kit (Clonetech) with nested primers. The first round of amplification was with primers nam2 and the Anchor primer (Clonetech). Part of this reaction (1/2 μl) was reamplified with *nam*–RACE primer (5′-dGTGATGGAGCTCTTCATCATCAGTTGGATGG-3′) and the Anchor primer. Independent *dTph1* insertion alleles were identified among 2000 Petunia plants by PCR with primers nam1 and the transposon-specific out-1 primer (5′-dGGGAATTCGCTCCGCCC CTG-3′), complementary to the terminal inverted repeat of *dTph1*, as described by Koes et al. (1995).

Insertion and footprint alleles were amplified by PCR, using either primers nam1 and nam3 (5′-dGCTCTAGATTTGCCACCATTTGAA GTG-3′) or nam1 and nam5 (5′-dGGGAATTCAAAGCTGCTGGCA CTC-3′) (Figure 5A), and appropriate subfragments were cloned into pBluescript-KS plasmids and sequenced.

Sequencing was performed using asymmetric PCR with fluorescent M13 primers, employing an Applied Biosystems DNA sequencer model 370A. All primers were synthesized by Isogen Bioscience.

Microscopical Sections

Flower buds and hand-dissected embryos were fixed, dehydrated, and embedded in paraffin as described by Cañas et al. (1994), and cut into 8 μm sections. Sections were then hydrated through a graded xylene/ethanol/water series and stained for a few minutes in toludine blue. After staining, sections were dehydrated through the reverse water/ethanol/xylene series and mounted in Entallan.

SEM

Young flower buds were dissected under a stereo microscope. Organs in outer whorls were removed by "cutting" them with a forceps, to view organs in the inner whorls. Samples were fixed in 3.7% formaldehyde, 5% acetic acid, and 50% ethanol for at least 16 hr, dehydrated in a graded ethanol series, and critical–point dried, using liquid CO_2. Flower buds were mounted on stubs with double-sided tape, sputter-coated with gold, and viewed in a JEOL JSM6400 scanning electron microscope, using accelerating voltages of 5–10 kV.

In Situ Hybridizations

Tissues (inflorescences with several flower buds or hand-dissected embryos) were fixed, dehydrated, and embedded in paraffin as described by Cañas et al. (1994), and cut into 8 μm sections. To generate a *nam*-specific probe, a 522 bp HdIII–KpnI fragment spanning the 3′ half of the *nam* cDNA was made blunt by Klenow polymerase and cloned into SmaI-digested plasmid pGEM3. Dygoxygenin-labeled RNA probes were obtained by T7 polymerase-driven in vitro transcription, according to the instructions of Boehringer Mannheim. RNA transcripts were partly hydrolyzed for 45 min. Hybridization and immunological detection were performed as described by Canas et al. (1994).

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GenBank Accession Numbers

Accession numbers for the sequences reported in this paper are as follows: X92204 (*nam* genomic sequence) and X92205 (*nam* cDNA).