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SHORT COMMUNICATION

Appendix: a novel type of homeotic mutation affecting floral morphology

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Summary

A mutant with altered floral morphology, designated Appendix, was observed among the progeny of a regenerated transgenic tobacco (*Nicotiana tabacum*) plant. Appendix flowers had small outgrowths resembling pistils on the tips of fertile anthers. No obvious morphological changes could be seen in any other part of the plant. A more careful analysis using histological methods and scanning electron microscopy revealed that the outgrowths were composed only of a style and a stigma and that these added organs can support pollen germination and pollen tube growth. Examination of anther ontogeny using scanning electron microscopy indicated that the anther style and stigma of the Appendix mutant developed from a small group of cells at the anther tip coincident with development of the carpel style and stigma. Genetic analysis indicated that this mutation is nuclear, recessive and linked to the 'transferred DNA' (T-DNA) inserted during the generation of transgenic plants using *Agrobacterium tumefaciens*. The Appendix phenotype does not correspond to any of the other types of floral homeotic mutations that have been described so far.

Introduction

The systematic study of floral organ homeotic mutants in *Arabidopsis thaliana* and *Antirrhinum majus* has identified a number of genes that are required to specify organ type during floral development (for reviews see, Coen and Meyerowitz, 1991; Haughn and Somerville, 1988; Schwarz-Sommer *et al.*, 1990). Most of these genes can be assigned to one of three different classes, each of which appears to be required by the plant to specify the proper organ type in two adjacent floral organ whorls. Class A is required for sepals and petals, Class B is required for petals and stamens and Class C is required for stamens and carpels. The overlapping domains of influence and the results from double mutant analyses suggest that these three gene classes act in combination to establish a unique identity for each of the organ whorls.

Several of the floral organ identity genes have been characterized in detail at the molecular level (*AG, AP3* genes from *Arabidopsis, DefA*$^+$ gene from *Antirrhinum*). Such studies have suggested that the *AG, AP3* and *DefA*$^+$ gene products are flower-specific DNA-binding proteins (Jack *et al.*, 1992; Schwarz-Sommer *et al.*, 1990, 1992; Sommer *et al.*, 1990; Yanofsky *et al.*, 1990). Furthermore, *in situ* hybridization studies (Bowman *et al.*, 1991; Drews *et al.*, 1991; Jack *et al.*, 1992; Schwarz-Sommer *et al.*, 1990; Yanofsky *et al.*, 1990) have indicated that transcript levels of the *AG, DefA*$^+$ and *AP3* genes are highest in the whorls that are affected when each of the genes is mutated. Thus, the products of the flower organ identity genes are probably proteins which regulate transcription and must themselves be regulated at the level of transcription.

The floral organ identity genes must direct primordial cells to follow an organ-specific programme of cell division and differentiation. Although it is not yet understood how these genes achieve such a complicated task, additional regulatory genes are probably involved. For example, the reproductive organs comprise a number of distinct suborgan structures (ovules, style, stigma, and ovary of the carpel; filament and anther of the stamen). The developmental programmes for such structures may be controlled by as yet unidentified regulatory genes under the direction of, or in combination with, the floral organ identity genes (Bowman *et al.*, 1991). In this paper, we describe a floral homeotic mutant of tobacco, Appendix, in which a group of cells at the tip of the anthers develop into a style and a stigma. Thus it appears that Appendix is defective in a gene required to specify the correct fate of only a subset of the cells in the developing anther.

Results

Mutant isolation and mature floral phenotype

A mutant with altered stamen development was discovered among the progeny of a tobacco plant (*N. tabacum* cv.

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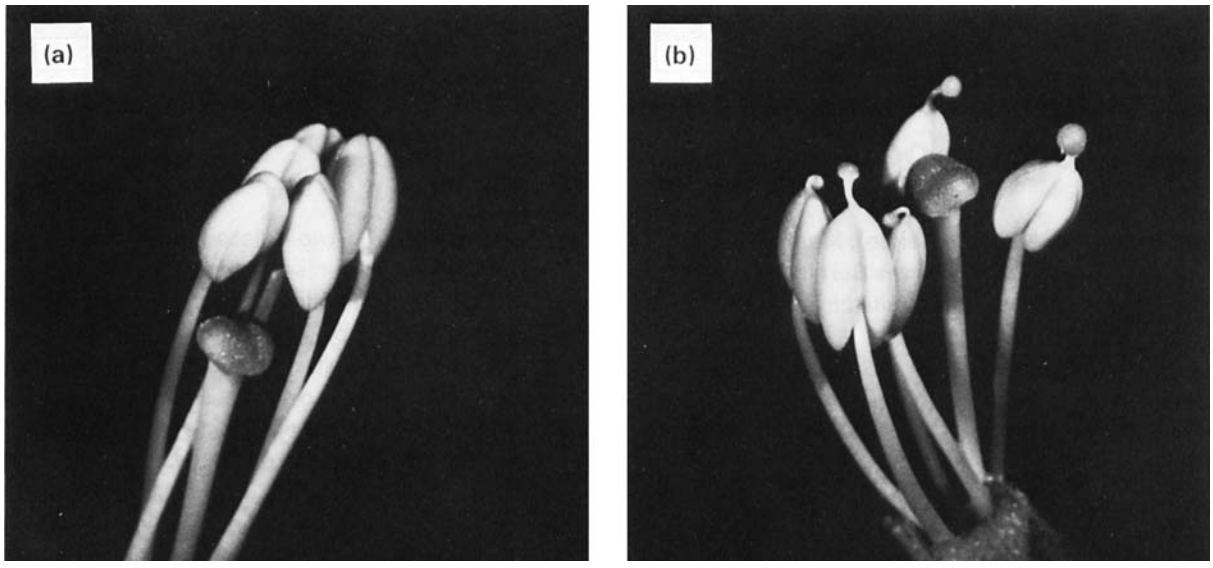

Figure 1. Normal and Appendix floral phenotypes.
(a) Normal tobacco flower, petals removed. (b) Appendix flower, petals removed.

Xanthi) regenerated from cells transformed using *A. tumefaciens* strain GV3111SE carrying a derivative of the pMON200 vector (see Experimental procedures). Other plants transformed with the same *Agrobacterium* strain did not show any mutant progeny. The mutant was named Appendix because of the presence of a small pistil-like outgrowth on the filament-distal tip on each anther as shown in Figure 1. Mutant stamens were otherwise normal in morphology and dehisced to release functional pollen grains. In addition, the morphology of other parts of Appendix (other floral organs, inflorescence, vegetative shoot) were not obviously different from wild-type tobacco. The mutant phenotype is due to a recessive allele (*appendix-1*) of a nuclear gene we designate *appendix*$^+$ (*apx*$^+$) (see Genetic analysis).

Light microscopy and scanning electron microscopy (SEM) were used to determine, more exactly, the nature of the pistil-like outgrowths of the anther. The results are presented in Figures 1–3. The general morphology of the anther outgrowths (Figures 1 and 2) is typical of wild-type style and stigma whereas the reminder of the Appendix stamen does not usually differ from wild-type stamens (Figure 2, compare a and b). The surface features of the epidermal cells of the anther style and stigma were typical of those of the wild-type style and stigma (compare Figure 2c with 2d and 2e with 2f). The presence of stigmatic papillae is especially worth noting (Figure 2f). Histological examination of normal style and stigma (Figure 3a) and of Appendix outgrowths (Figure 3c) indicated the similarity in anatomy. More specifically, the presence of stigmatic papillae and of a darkly staining region typical for transmitting tract cells can be noted in Figure 3c. A close examination of the junction between the anther and the style (Figure 3d, compare with 3b: normal anther) did not reveal the presence of any ovarian structures (e.g. locules, ovules, placenta) or cell types. So, structurally the outgrowths in the Appendix mutant represent a style and a stigma rather than an entire pistil.

Three other characteristics about the Appendix outgrowths are worth emphasizing. First, the anther stigmas consist of a single lobe rather than the two lobes of the wild-type bicarpellate pistil (Figures 2 and 3). Secondly, although morphologically similar to a style and stigma, the Appendix outgrowths are considerably smaller than those of a normal pistil: the normal stigma has a diameter of 2 mm and the total length of the style and stigma is about 35 to 40 mm whereas the anther stigmas are only 0.6 mm in diameter and the outgrowth lengths range from 1.5 to 2 mm. This difference in size is mainly due to a difference in cell number between the two structures as can be seen in Figure 3 (compare a and c). Thirdly, the Appendix anther outgrowths were shown to be functionally equivalent to a stigma and style. As on a normal stigma, germination of pollen and penetration of the stigmatic surface by the pollen tube occurred at a high frequency. In addition, the pollen tubes grew basipetally through the top half of the transmitting tract-like tissue (results not shown).

About one-tenth of the Appendix plants (in the T_1 or the T_2 generation) had one or a few anthers displaying an abnormality other than the style and stigma outgrowth. These abnormalities were characterized by the presence of green tissue emerging from the connective tissue or by pink, petal-like tissue emerging from the anther or the filament. It is important to point out that these abnormalities

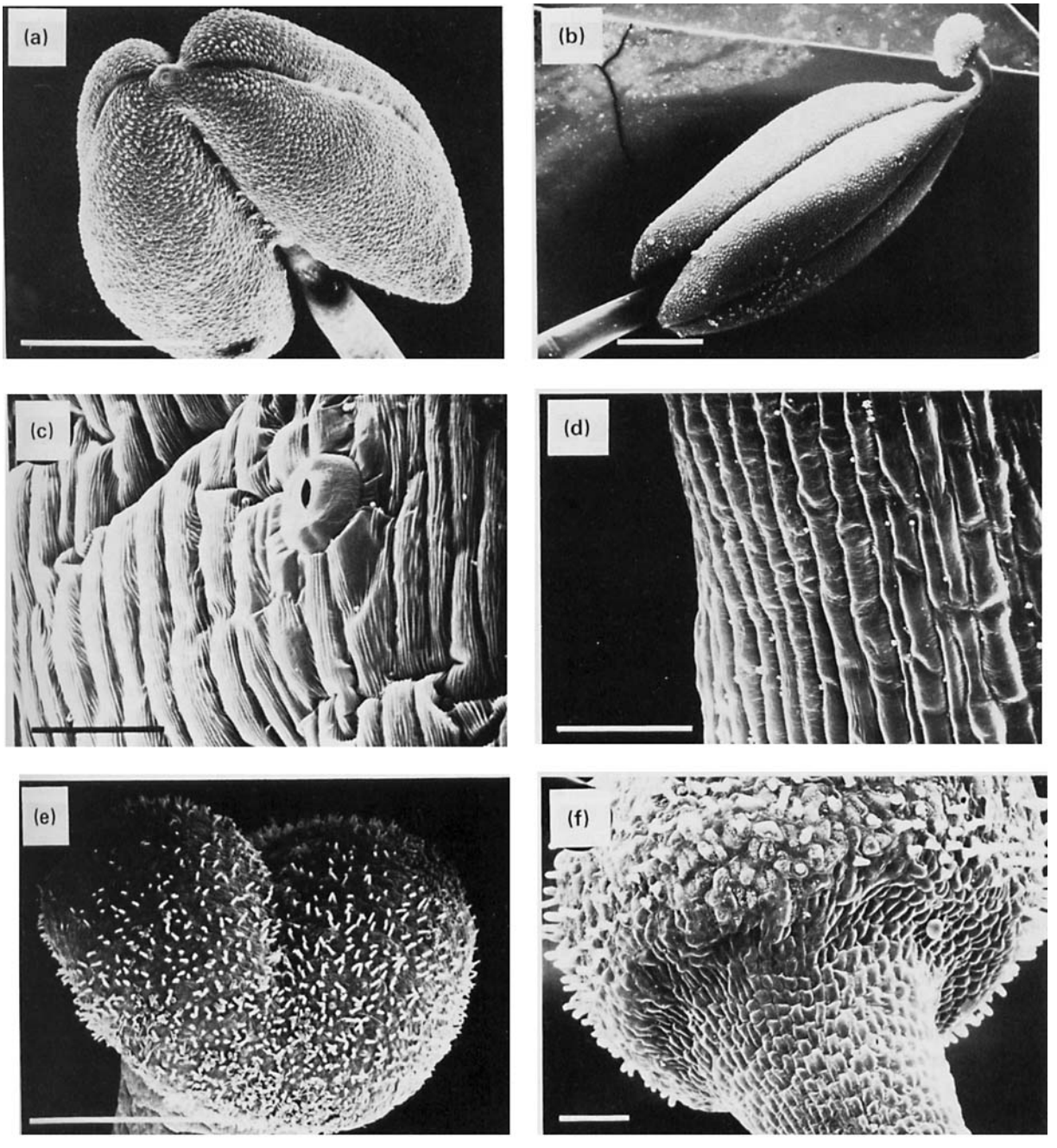

Figure 2. Scanning electron micrographs: mature stamens and cell surface features of mature floral organs.
(a) Wild-type stamen. (b) Appendix stamen. (c) Wild-type style cells. (d) Appendix style-like cells. (e) Wild-type stigma cells. (f) Appendix stigma-like cells. Bar = 1 mm (a, b and e), 0.1 mm (f), 0.05 mm (c and d).

usually arise on only one of the anthers of a given flower: the others display the usual Appendix phenotype. Also, such abnormal anthers were never observed in our wild-type tobacco plants. We do not understand the relation between these abnormalities and the Appendix phenotype nor the reason why only one anther in a given flower, and also only one (or a few) flower in a plant is affected.

Ontogeny

The analysis, by SEM, of flower buds harvested at different stages of development indicated that the Appendix mutant first deviated from wild-type when the gynoecium began to differentiate a style and stigma (stage −4 according to Koltunow *et al.*, 1990; see Figure 4a–c). At this stage the

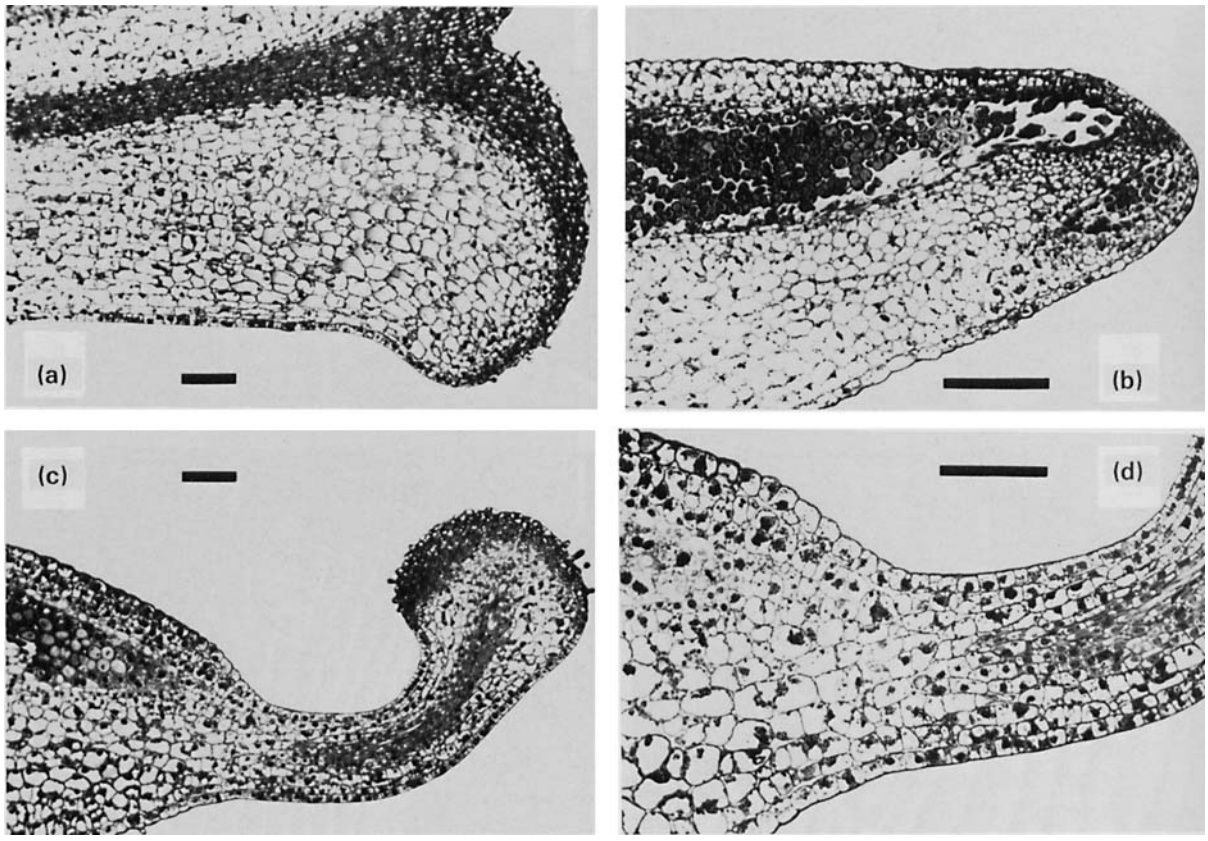

Figure 3. Histological examination of normal and Appendix floral organs.
(a) Normal tobacco style and stigma, stage +3 (about 5 days before flower opening). (b) Normal tobacco anther, stage +6 (about 3 days before flower opening). (c) Appendix style and stigma outgrowth showing the junction with the anther, stage +6. (d) Close-up view of the outgrowth–anther junction, stage +6. Bar = 0.1 mm.

Appendix outgrowths started to develop from a small group of cells at the tip of the anther (Figure 4c) and differentiated into a style and a stigma (Figure 4d–g) in parallel with the gynoecial style and stigma (data not shown). The same group of cells in the wild-type anther are distinct from surrounding cells but, unlike Appendix anthers, develop little past stage −4 (compare Figure 4a with 4h).

Genetic analysis

The Appendix mutant was analysed genetically to determine the nature of the mutation and its relationship to the T-DNA. The original transformant (T_0) had wild-type morphology. Fifty-six selfed progeny (T_1 generation) had either wild-type (42) or Appendix (14) floral phenotype (ratio of 3:1, $\chi^2 = 0$, $P = 1.00$) suggesting that the T_0 plant was heterozygous for a recessive allele (*apx-1*) of the *apx*$^+$ gene.

Southern blot analysis (data not shown) suggested that the kanamycin-resistant T_0 plant carried a single integrated copy of the T-DNA (marked with the *NPT-II* gene). As expected, the T-DNA was found to segregate like a single dominant nuclear locus in the T_1 generation (14 kan^R/kan^R: 31 kan^R/kan^S: 11 kan^S/kan^S, $\chi^2 = 0.97$, $P > 0.75$). Furthermore, all 14 T_1 plants homozygous for kan^R had Appendix phenotypes while T_1 plants heterozygous for kan^R (31) or homozygous for kan^S (11) had wild-type floral phenotypes. These data indicate that the T-DNA and the *apx* gene are genetically linked.

The linkage between the T-DNA and the *apx* gene was further confirmed by crossing T_1 plants homozygous for the T-DNA insertion (kan^R/kan^R, Appendix phenotype) with T_1 plants heterozygous for the T-DNA insertion (kan^R/kan^S, wild-type floral phenotype). Among 57 F_1 progeny examined, parental phenotypes were recovered in a ratio of 1:1 (27 kan^R/kan^S, wild-type flowers: 30 kan^R/kan^R, Appendix flowers, $\chi^2 = 0.16$, $P > 0.9$) while no recombinant progeny were observed. The results above are significantly different from those expected if the T-DNA and the *apx* gene were linked by 14 map units (eight recombinant progeny in 57 plants: $\chi^2 = 9.4$, $P < 0.05$) suggesting that the T-DNA and *apx* are within 14 map units of each other.

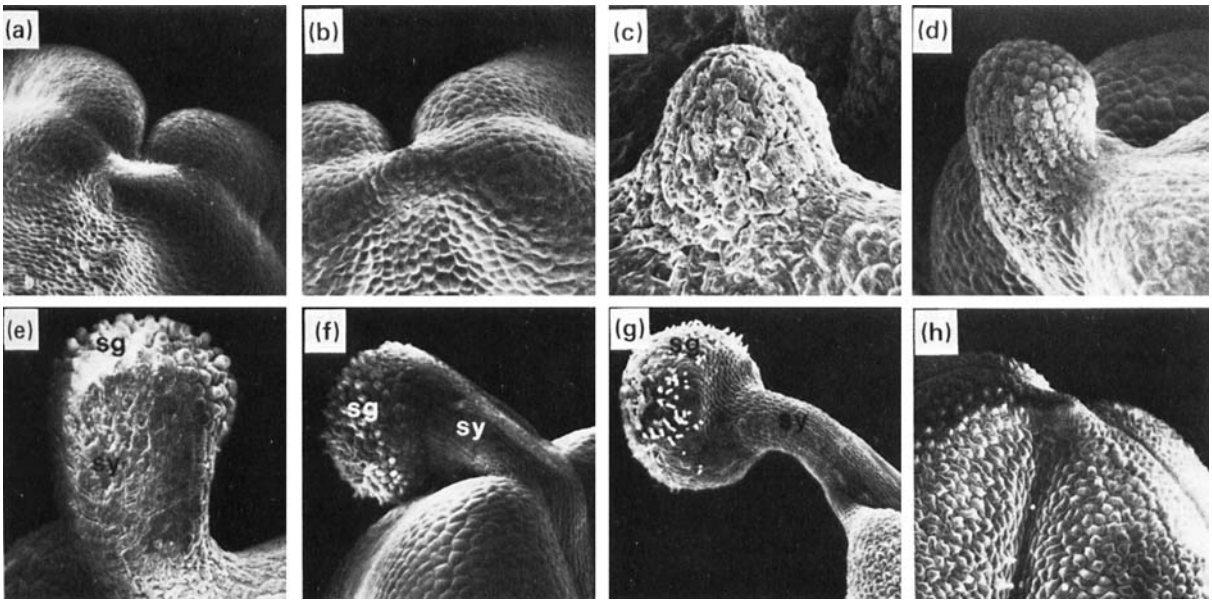

Figure 4. Scanning electron micrographs of the filament-distal tip of developing anthers in wild-type and Appendix mutant flowers.
Tobacco flower development has been divided into 19 stages as described previously (Koltunow *et al*., 1990) where the first seven stages (−7 to −1) represent the period in development prior to the formation of tetrads in the anther (stage +1) and where stage +9 occurs about 2 days before dehiscence. Perianth organs have been removed after fixation to expose the reproductive organs.
(a) Wild type stage −5 anther, magnification ×107. (b) Appendix stage −5 anther, magnification ×206. (c) Appendix stage −4 anther, magnification ×480. (d) Appendix stage −3 anther, magnificatlon ×252. (e) Appendix stage −3 anther, magnification ×228. (f) Appendix stage +1 anther, magnification ×115. (g) Appendix stage +9 anther, magnificaton ×44. (h) Wild-type stage +9 anther, magnification ×69. sy, style; sg, stigma.

Discussion

We have described a tobacco mutant where a functional style and stigma appears to develop from a group of cells at the filament-distal tip of an immature (stage −4) anther. The ontogeny and time of development of the anther stigma and style are similar to the development of the normal style and stigma from cells at the tip of immature carpels.

Recent work on floral homeotic mutants in *Antirrhinum* and *Arabidopsis* has provided a conceptual framework for considering floral homeotic changes (Coen and Meyerowitz, 1991; Haughn and Somerville, 1988; Schwarz-Sommer *et al*., 1990). Three classes of genes with overlapping domains of expression function together to specify floral organ type. For example, Class B genes specify petals in combination with Class A genes and stamens in combination with Class C genes. A loss of function mutation in a Class B gene results in stamen to carpel transformations (because Class C alone specifies carpels) and petal to sepal transformations (because Class A alone specifies sepals). At least some and perhaps all of the Class A, B and C homeotic genes are expressed throughout floral organ development (Bowman *et al*., 1989, 1991) and may be required to control other downstream regulatory genes needed to direct specific aspects of morphogenesis within a given organ.

Like the Class B genes, a recessive mutation in *apx*$^+$ results in a stamen to carpel homeotic change. However, only the cells at the tip of the anther are affected. If we assume that the floral organ identity in tobacco is regulated in a manner similar to *Antirrhinum* and *Arabidopsis*, how can the role of *apx*$^+$ with respect to Class A, B and C genes be rationalized? Several possibilities exist if we assume that the *apx-1* allele results in a loss of *apx*$^+$ function. First, the *apx*$^+$ gene product under the positive control of the Class B genes could be required to direct the anther tip cells to adopt a stamen-specific developmental fate. Secondly, the *apx*$^+$ gene product may be needed to activate the late expression of one or more of the Class B genes in the anther tip cells of state −4 stamens. Thirdly, *apx-1* may be a mutation in a Class B gene promoter element needed for expression of the Class B gene in the group of cells at the tip of an anther. Finally, *apx-1* could represent a weak gain-of-function mutation, expecially considering that *N. tabacum* is an allotetraploid. If so, *apx-1* may be an allele of a Class C gene resulting in its overexpression in the anther tip cells.

The phenomenon of stigmatoidal outgrowths from stamens has been observed previously. Both genetic and environmental factors are known to cause such developmental abnormalities. Plants regenerated from a mutant tobacco cell line resistant to the S-adenosylmethionine decarboxylase inhibitor, methylglyoxal-bis(guanylhydra-

zone), (Mgr9; Malmberg and McIndoo, 1984) were found to have stigmatoid outgrowths on sterile anthers in addition to carpel abnormalities (Malmberg *et al*., 1985). *In vitro*, culture of wild-type tobacco stamen primordia show a tendency to produce pistil-like outgrowths (Hicks, 1979). Finally, a mutant with a phenotype closely resembling Appendix, also due to a recessive mutation, has been described briefly (White, 1914). Unfortunately, to our knowledge, seeds are no longer available to test the genetic relationship of this mutant to Appendix.

It is also interesting to note that both stigmatoid and petaloid stamens are among a variety of floral abnormalities caused by a mitochondrial mutation in some male-sterile tobacco cultivars (Kofer *et al*., 1991). The relationship between the mitochondrial mutation and *apx*$^+$, if any, is as yet unclear.

Experimental procedures

Tobacco transformation and growth conditions

A chimeric gene consisting of part of the α′ subunit of β-conglycinin (a soybean seed storage protein) promoter fused to the GUS reporter gene was introduced into the pMON200 vector (Sanders *et al*., 1987). This construct was introduced into the *A. tumefaciens* strain GV3111SE for transformation of tobacco according to the standard procedure (Horsch *et al*., 1985).

Tobacco plants were grown either at 22°C under constant illumination or in the greenhouse at temperatures ranging from 22 to 28°C with a 16-h photoperiod.

Histological examination

The floral organs were placed in a fixative (3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 24 h at 4°C. They were then rinsed twice in the buffer and allowed to fix for 1 h in 1% osmic acid in cacodylate buffer. After two washes in buffer, the tissues were dehydrated through a graded ethanol series and infiltrated with Jembed 812 resin for 3 days at 60°C. Two-micrometer sections were cut and stained with 0.1% toluidine blue, 2.5% Na_2CO_3.

Scanning electron microscopy

For SEM, mature or developing flowers were vacuum infiltrated with 3% glutaraldehyde in 0.02 M sodium phosphate buffer (pH 7.2) and fixed in the same solution overnight at 4°C. Samples were then rinsed in buffer and dehydrated through a graded acetone series at 4°C, before critical point drying in liquid carbon dioxide and mounting on stubs. Perianth organs were removed from very young flowers using pulled glass needles. Flowers were then coated with gold in an Edwards S150B sputter coater and examined with a Philips 505 scanning electron microscope at an accelerating voltage of 30 kV.

Pollination studies

Wild-type pollen was used to hand pollinate anther stigmas as well as pistils of wild-type flowers. After 6 h (for SEM) or between 6 and 24 h (for squashes of anther outgrowths) the tissue was examined. SEM methods have already been described (see above). For squashes, Appendix anthers were squashed onto a slide, stained with aniline blue and examined under a fluorescent microscope.

Germination of the seeds on kanamycin

The ability of the seeds to germinate and of the plantlets to grow in the presence of kanamycin was used to determine if the *NPT-II* gene was present in the parental plant and, if so, whether it was homozygous or heterozygous, as described by Paszkowski and Saul (1988).

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