Genetic ablation of petal and stamen primordia to elucidate cell interactions during floral development

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SUMMARY

Two models have been proposed to explain the coordinated development of the four whorls of floral organs. The spatial model predicts that positional information defines the four whorls simultaneously, and that individual organs develop independently of surrounding tissues. The sequential model suggests that inductive events between the outer and inner whorl primordia are required for appropriate organogenesis. To test these models we have genetically ablated second and third whorl floral organ primordia to determine if organ identity, number or position are perturbed in the first or fourth whorls. We used diphtheria toxin to specifically ablate floral cells early in development in Nicotiana tabacum and in Arabidopsis thaliana. Second and third whorl expression of the diphtheria toxin A chain coding sequence (DTA) was conferred by the Arabidopsis APETALA3 (AP3) promoter. Both Nicotiana and Arabidopsis flowers that express the AP3-DTA construct lack petals and stamens; it appears that the second and third whorl cells expressing this construct arrest early in floral development. These results show that first and fourth whorl development is normal and can proceed without information from adjacent second and third whorl primordia. We propose that positional information specifies the establishment of all four whorls of organs prior to the expression of AP3 in the floral meristem.

Key words: Arabidopsis, Nicotiana, diphtheria toxin, ablation, pattern formation

INTRODUCTION

Floral organ primordia arise from the coordinated divisions of cells on the flanks of the floriually determined shoot apical meristem. In response to floral inductive signals, the meristic apex generally becomes more domed, and increases in mitotic activity (Steeves and Sussex, 1989). The first whorl of floral organ primordia arising from the dividing cells on the flank of the apex gives rise to sepals. Generally, the subsequent organ primordia initiate from outer to inner whorls, and differentiate as petals, stamens and carpels.

It appears that the mechanisms specifying floral organ identities are distinct from those specifying number and position of floral organ primordia. Floral homeotic mutants in Antirrhinum and Arabidopsis alter the identity of organs, but do not generally perturb the number or position of organ primordia within a whorl (Meyerowitz et al., 1991). Other floral mutations disrupt organ number, but otherwise have no obvious effect on floral development (Clark et al., 1993).

There are two models that attempt to describe how the cells of the floral meristem interact to initiate organ development. Holder (1979) proposed that positional signals are laid down simultaneously in the meristem, and that floral organs then differentiate independently of one another. In contrast, Wardlaw and others (Wardlaw, 1957; Heslop-Harrison, 1964; Hicks and Sussex, 1971) suggested that sequential inductive signals were required to specify the floral pattern. Inductive signals from the outer (first) whorl were proposed to trigger the initiation of the second whorl; development of the second whorl allowed the production of a second signal which induced development of the third whorl, and so on. An important difference between this sequential model and the spatial model is that development of a particular organ in the sequential model is dependent on signals from the previous whorl.

A number of surgical experiments have been performed on floral meristems in an effort to distinguish between these models. Most of these surgical experiments have been interpreted as supporting a sequential model. Excisions of individual whorls were shown to affect the normal development of Nicotiana flowers grown in sterile culture (McHughen, 1980). Removal of stamen primordia significantly inhibited carpel development and was presented as evidence to support the sequential model. Floral meristems of different species have been bisected and all showed the potential to regenerate two flowers if the bisection was performed prior to the initiation of sepal primordia (Cusick, 1956; Hicks and Sussex, 1971; Soetiarto and Ball, 1969). These observations showed that cells in the floral meristem were not irreversibly fated to give rise to a particular organ. Bisections of floral meristems at successive stages indicated that the developmental potential of regenerating flower buds became limited with time. For example, if meristem bisections were performed after sepal primordia are
initiated, no sepalos regenerated on the operated side of the apex. Such information suggested that a developing flower had a series of organ initiation phases. Based on these observations, Hicks and Sussex (1971) proposed a refined version of the sequential model. Since a subtending whorl (e.g., sepalos) was not required on the operated side of the apex to initiate the next whorl (e.g., petals) and the pattern of regeneration was organized around the new apex, they suggested that the inductive signal was not derived from the extant organ primordia but from the center of the floral apex. While these surgical experiments have been useful in studying cell interactions in the floral meristem, it is impossible to reliably remove all the cells of an organ primordium. In addition, other cells are damaged during the manipulation of the floral meristem.

More efficient ways of removing cells with the smallest possible impact to the system have superseded traditional surgical techniques. Lasers have been used to specifically kill individual cells in Caenorhabditis elegans (Kimble, 1981; Sulston and White, 1980) as well as in plants (Croxdale et al., 1992). Another useful technique is genetic cell ablation (Bellen et al., 1992; Breitman et al., 1987; Palmiter et al., 1987). The success of this technique relies on the use of a tissue-specific promoter to express a cell-autonomous toxic gene product in the cells to be removed. This approach has been used successfully in plants using the toxic products of the diphtheria toxin A chain gene (DTA) (Thorsness et al., 1993) or the barnase gene (Mariani et al., 1990; Goldman et al., 1994). The DTA toxin is cell-autonomous since without the diphtheria toxin B chain and extracellular targeting sequences, it cannot be transported across the plasma membrane (Pappenheimer, 1977). DTA functions by ribosylating the EF2 translation initiation factor and consequently inhibiting all protein translation (Collier, 1975). Cells expressing DTA are unable to maintain protein synthesis, cannot divide, and eventually die.

The experimental approach we have taken is to use genetic ablation with DTA to remove the second and third whorl cells in the floral meristem at an early stage in development. The removal of two whorls from the floral meristem allows us to make predictions about organ formation based on the two models of floral development. If the spatial model is correct, we would expect the first whorl of sepals and the fourth whorl carpels to develop normally. Conversely, the sequential model would predict disruptions in fourth whorl and/or first whorl carpels to develop normally. We propose that the inductive signal was not derived from the extant organ primordia but from the center of the floral apex.

In situ hybridisation
Preparation of the Nicotiana tissue for in situ hybridisation was carried out as described by Jackson (1991). An antisense RNA probe of NAG1 was prepared from the pKY60 plasmid (Kempin et al., 1993). The plasmid was linearised with BglII, which removed the MADS box region and was transcribed with T7 polymerase (Boehringer Mannheim Corp., Indianapolis, IN). RNA probes were labelled with digoxigenin-UTP (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer’s instructions. 8 µm paraffin sections of transgenic Nicotiana buds were affixed to Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA). Pretreatment of slides, hybridisation, and washing were carried out according to the method of Langdale et al. (1988) and (Jackson, 1991) with slight modifications. The digoxigenin label was immunologically detected using anti-digoxigenin-alkaline phosphatase, and nitroblue tetrazolium and X-phosphate to detect alkaline phosphatase activity according to the manufacturer’s instructions (Boehringer Mannheim Corp., Indianapolis, IN).

MATERIALS AND METHODS

Plant material
Nicotiana tabacum cv SR1 plants were grown at 28°C under standard greenhouse conditions. Arabidopsis thaliana ecotype NoO were grown in a growth room at 22-24°C under a 16 hour day/8 hour night regime, with lights at 175 µmol m⁻² sec⁻¹ at the pot top.

RESULTS

Construction of a chimeric DTA toxin
A prerequisite for the success of this experiment is a highly tissue-specific promoter which restricts the expression of the toxin to cells in the second and third whorl. We chose to use

Strains and molecular cloning
Escherichia coli strain DH5α was used to grow plasmids by standard procedures (Sambrook et al., 1989). Agrobacterium tumefaciens strain LBA4404 (Clonetech Laboratories, Inc., Palo Alto, CA) was used for plant transformation.

The chimeric AP3-DTA construct was made by subcloning the DTA promoter (Irish and Yamamoto, 1995) to drive the expression of the DTA gene (Mariani et al., 1990; Goldman et al., 1994). The AP3 gene was subcloned into pKSADN (Thorsness et al., 1993) or the barnase gene (Bellen et al., 1992; Breitman et al., 1987; Palmiter et al., 1987). The AP3 gene is expressed in the floral meristem. We discuss the potential use of this construct for studies on floral development.

Plant transformation
Transgenic Nicotiana plants were produced by leaf disc transformation (Horsch et al., 1988) using Agrobacterium tumefaciens transformed with pBIADN. Transgenic Arabidopsis plants were produced by root transformation using a similar protocol to that of Valvekens et al. (1988). Transgenic Arabidopsis lines were all primary transformants since the transgene could not be transmitted to the next generation.

Histology and microscopy
Tissue was fixed using FAA (3.7% formaldehyde, 50% ethanol, 5% acetic acid) by vacuum infiltration for 30 minutes and dehydrated in a graded ethanol series. Nicotiana flowers were embedded in paraffin and cut into 8 µm sections. Sections were hydrated and stained in DAPI, 1 µg ml⁻¹, for 30 minutes and visualised with fluorescence optics. Arabidopsis flowers were embedded in SPURRS resin and cut into 2 µm sections, then stained with a solution of 0.05% toluidine blue, 1% sodium borate.

For scanning electron microscopy, dehydrated flowers were critical point dried in liquid CO₂. Specimens were sputter coated with gold palladium and examined in an ISI SS40 scanning electron microscope with an accelerating voltage of 10 kV.

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the promoter of the *Arabidopsis AP3* gene, which is expressed in petal and stamen primordia (Jack et al., 1992). *AP3* transcripts can first be detected at stage 3 (stages according to Smyth et al., 1990) in eight to ten cells in all three cell layers of the floral meristem.

We used a 1.9 kb *AP3* genomic clone to construct a translational fusion between the *AP3* promoter and the *DTA* coding region (Fig. 1). The genomic clone included 1.7 kb of 5′ sequence, which is sufficient to confer normal *AP3* expression in *Arabidopsis* (Irish and Yamamoto, 1995), and the first 162 bp of the *AP3* open reading frame.

**Expression of the chimeric AP3-DTA toxin in Arabidopsis specifically ablates second and third whorl organs**

The primary transgenic *Arabidopsis* plants that expressed the chimeric DTA protein had a striking phenotype which consisted of a normal flower with no petals or stamens (Fig. 2). Southern analysis of one line, with probes from the right and left border of the T-DNA insert, showed that there were up to five copies of the chimeric gene inserted at three locations in the genome (data not shown). Five other independent transgenic lines were obtained which displayed similar phenotypes.

The transgenic *Arabidopsis* flowers were sectioned to determine the morphology at various stages of development. The mature transgenic flowers had two distinct whorls of organs which were almost identical to those found in the first and fourth whorls of the wild type (Fig. 2C,D). The first whorl contained four sepals which appeared normal at the morphological and histological level. The remaining organs consisted of two fused carpels which appeared normal, although the

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Fig. 1. Schematic diagram of the AP3-DTA chimeric toxin. The white box represents *AP3* sequence; the shaded box represents DTA sequence. Hatched line indicates the nopaline synthase transcription terminator sequence. The amino acid sequence of the translational fusion between AP3 and DTA is indicated below the diagram.

Fig. 2. Developmental stages of wild-type and transgenic *Arabidopsis* flowers. Comparisons of wild-type (A,C,E,G,I) with transgenic *Arabidopsis* flowers expressing the chimeric AP3-DTA construct (B,D,F,H,J). (A) A mature wild-type flower. (B) The transgenic *Arabidopsis* flower (black arrow indicates a sepal); two first whorl sepals have been removed (white arrow indicates the scar) to reveal the gynoecium in the transgenic flower. (C) A longitudinal section of a stage 11 wild-type flower. (D) A transgenic *Arabidopsis* flower at a similar stage to (C). (E,F) Longitudinal sections through the gynoecium at late stage 12; compare ovule development between (E) wild-type and (F) the transgenic *Arabidopsis*. In F some ovules did not fully develop integuments. (G,H) Longitudinal sections through flowers at stage 3 were similar for (G) wild-type and (H) the transgenic *Arabidopsis*. (I,J) By stage 6 the organ primordia were clearly visible in wild-type (I) and absent in the transgenic (J) *Arabidopsis*. Scale bars, C,D, 100 μm; E,F, 1, 50 μm; G,H, 25 μm.
ovules occasionally displayed an aberrant structure which is discussed below. The epidermal cell layer of the sepals and the gynoecium are adjacent and there is no apparent scar tissue from the absent second and third whorls (Fig. 2D). From the phenotype of these transgenic lines we conclude that in Arabidopsis two whorls, the second and third in wild-type flowers, have been specifically ablated and that this ablation does not cause any significant defect in the development of the first or fourth whorls.

**Second and third whorl cells are ablated early in development**

*AP3* transcripts are first detectable at the stage when sepal primordia are just beginning to initiate (Jack et al., 1992). We sectioned flower buds to determine when we could detect the onset of morphological anomalies. At stage 3 there are no obvious differences between wild-type and transgenic flowers (Fig. 2G,H). By stage 6, wild-type flowers had apparent stamen and petal primordia (Fig. 2I). A section from the equivalent stage in the transgenic flower bud lacked petal and stamen primordia (Fig. 2J). This implies that the cells that are normally determined to become organs in the second and third whorls are ablated early in development. Later, at stage 7, after the carpels have started to initiate, the wild-type flower has a distinct pattern of four whorls of organs. At a similar stage, the transgenic flowers had only two whorls of organs. In addition, sepal and carpel epidermal cells were in contact at the base of the organs (data not shown). If inductive signals are exchanged between these two whorls they do not alter organ development since these primordia develop into essentially normal organs.

**Ovule development is aberrant in transgenic Arabidopsis flowers**

In wild-type carpels an outer and inner integument grow to surround the ovule (Robinson-Beers et al., 1992). In the transgenic Arabidopsis flowers, however, some of the ovules did not appear to have normal integuments (Fig. 2F). This phenotype may be due to specific cell ablation or to an inductive effect due to the loss of petals and stamens. Specific cell ablation is more consistent with the known

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**Fig. 3.** Genetic ablation of the petals and stamens in two different transgenic lines of Nicotiana expressing the chimeric AP3-DTA construct. (A) Wild type, longitudinally split to show inner organs. (B) Longitudinally split transgenic Nicotiana flower displaying the strong phenotype has an essentially normal gynoecium despite the absence of petals and stamens. (C) A patch of stamen-like tissue on an otherwise normal gynoecium from a transgenic, strong phenotype, Nicotiana. (D) Wild-type gynoecium (far left) compared with typical gynoecium from the transgenic, strong phenotype, Nicotiana with the outer organs (sepals, petals and stamens for the wild type; sepals for the transgenic Nicotiana) removed for clarity. Of the flowers that had carpels, 15% had a small mosaic sector which caused some distortion of the gynoecium (second from left). 23% had more severe distortions (third from left), while 18% had up to two mosaic sectors which resulted in severe distortion of the carpels and an inhibition of the style fusion and elongation (fourth from left). 44% of the gynoecia were essentially normal, similar to that in B. (E) Representative flowers from transgenic, intermediate phenotypes. The severity of the phenotype increases from left to right.
expression pattern of AP3, since AP3 is expressed late in pistil development at the base of the integument (Jack et al., 1992). Not all of the integuments were ablated in the transgenic flowers. This partial effect may be due to weak AP3 expression in this tissue, which lowers the amount of chimeric toxin below a threshold required for toxicity. While in other systems the DTA toxin has proved to be very potent (Yamaizumi et al., 1978), either the construct or the position of the transgene insert may have provided a low level of DTA toxin, resulting in a threshold effect. The existence of such a toxic threshold was also suggested by our studies with transgenic Nicotiana. Therefore, the loss of some ovule integuments was likely to be due to specific cell ablation rather than to an inductive effect resulting from petal and stamen ablation.

When the transgenic Arabidopsis flowers were pollinated with wild-type (Landsberg erecta) pollen some seed developed to maturity. None of the seed we recovered were kanamycin resistant, which suggested that the AP3-DTA construct was also expressed gametophytically or in the embryo.

Expression of the chimeric AP3-DTA toxin results in loss of second and third whorls in Nicotiana

We also used the AP3-DTA construct to transform Nicotiana tabacum. The primary transformants of Nicotiana we generated had a variety of phenotypes which appeared to depend on the copy number of T-DNA inserts in the genome. As with Arabidopsis, the inserts were characterised by Southern blot analysis using right and left border probes specific for the T-DNA insert (data not shown). The strongest phenotype correlated with three inserts; an intermediate phenotype with two transgene inserts; and weak phenotypes appeared in two lines containing one insert each.

In the transgenic Nicotiana line with the strong phenotype, the petals and stamens are absent and the phenotype is quite similar to that seen with Arabidopsis (Fig. 3B). The first whorl has an essentially normal calyx (fused sepals) with an inner whorl of carpels which are fertile when pollinated with wild-type pollen. The calyx occasionally contains four fused sepals, as compared to five in wild type, and the mature sepals are usually slightly longer (Fig. 3B). No petals or stamens can be detected. Unlike transgenic flowers from Arabidopsis, however, carpel development in the transgenic Nicotiana flowers is delayed compared to wild type (Fig. 4). Although the outgrowth of the carpels is significantly delayed, eventually these carpels develop to full size. The transgenic Nicotiana flowers also occasionally displayed defects in the final morphology of the gynoecium. Occasionally, what appeared to be mosaic patches of petal or stamen tissue developed on the carpels (Fig. 3C). One explanation for the appearance of these patches of petaloid or stamenoid tissue is that expression of the chimeric toxin is below a required threshold level in these cells. In addition, the gynoecia produced in this transgenic line were occasionally kinked or wrinkled (Fig. 3D).

We have shown that carpel development in transgenic Nicotiana with the strong phenotype is delayed (Fig. 4). This may be due to the delay or loss of an inductive signal to activate

Fig. 4. Delayed development of the gynoecium in the transgenic, strong phenotype, Nicotiana. Development of wild-type Nicotiana (A-C) compared with transgenic, strong phenotype, Nicotiana (D-F) flowers. Scale bars, 100 μm.
the organ identity genes in the fourth whorl. NAG1 is specifically expressed in the third and fourth whorls of wild-type floral meristems and is required for organ identity and determination in the floral meristem (Kempin et al., 1993). We used nRNA in situ analysis to determine if NAG1 is expressed in the retarded fourth whorl primordia of transgenic *Nicotiana* buds displaying the strong phenotype. We find that the NAG1 transcript is expressed in the fourth whorl during the period that the carpel growth is delayed (Fig. 5C). Expression of NAG1 in the carpels at this stage suggests that the delay in carpel development is not due to the loss of an inductive signal from the petals or stamens.

The transgenic *Nicotiana* line that displayed the intermediate phenotype showed flower phenotypes ranging from almost wild type, to having only one or two petals and stamens (Fig. 3E). The number of petal and stamen primordia that developed was variable and the organ fusions appeared disorganised. The petals were often misfused at their margins allowing petalloid segments to develop outside the corolla. When a petal was absent, fusion still occurred at the base of the corolla but rarely continued into the petal lobe, resulting in a slit corolla with one to four petals. Occasionally we have seen a complete corolla form from the fusion of only four petals. We rarely observed half petals. The temporal development of the gynoecium was similar to wild type in these flowers.

The transgenic *Nicotiana* plants that displayed a weak phenotype generally had wild-type flowers. Occasionally, abnormal fusions of the corolla were observed.

**Cell death induced by the transgene can be followed using DAPI**

One way to visualise cell death is to look for degradation of chromosomal DNA. In mammalian cells, DTA is one of the inhibitors of protein synthesis, which can cause characteristic internucleosomal fragmentation of DNA (Kochi and Collier, 1993; Martin, 1993). To determine if we can detect apoptotic-like cell death we stained young *Nicotiana* floral buds with DAPI to visualise DNA. Wild-type *Nicotiana* buds display a characteristic pattern of nuclear DAPI fluorescence (data not shown). Transgenic *Nicotiana* buds displayed much brighter DAPI fluorescence in cells between the first and fourth whorl (Fig. 5A,B). In addition, this fluorescence appeared to be distributed throughout the cytoplasm. Similar bright, diffuse DAPI fluorescence has been observed in cells undergoing apoptotic cell death (Kulkarni and McCulloch, 1994). We interpret the pattern of fluorescence we see as resulting from cells that are dying due to expression of DTA.

**DISCUSSION**

**Loss of petal and stamen primordia has little effect on the development of other floral organs**

The spatial model predicts that positional information specifying the floral pattern is set early in development and implies that the development of an organ is independent of the surrounding tissues. The phenotype of the transgenic flowers we have generated in *Arabidopsis* correlates with these predictions since sepal and carpel development were essentially unaffected by the removal of petal and stamen primordia. Although the transgenic *Nicotiana* plants displaying the strong phenotype occasionally showed a variety of defects in carpel development, the fact that these plants frequently produce a normal and functional gynoecium indicated that information from the second and third whorl primordia was not necessary for carpel development. We have shown that cell ablation occurs early, just after sepal primordia become apparent, in both *Arabidopsis* and *Nicotiana* (Figs 2I,J, 4). In these ablated flowers, the number and position of first and fourth whorl organs are normal. Since essentially normal first and fourth whorl development is observed in both *Nicotiana* and *Arabidopsis*, we propose that growth centers defining the position of the organ primordia in all four whorls are set up before the expression of the chimeric toxin in the second and third whorls. This positional information is sufficient to autonomously direct the development of floral organ primordia, consistent with the predictions of the spatial model.

Analysis of floral homeotic mutants in several systems has shown that the specification of organ identity in a particular whorl is not dependent on the identity of the previous whorl (Meyerowitz et al., 1991; Schwarz-Sommer et al., 1990; van der Krol and Chua, 1993). Therefore, the specification of organ identity has previously been considered to be consistent with a spatial model (Bowman et al., 1991; Haughn and Somerville, 1988). Since these homeotic mutants do not generally disrupt organ number or position, it appears that the mechanisms specifying floral organ identity are distinct from those specifying organ number and position (Meyerowitz et al., 1991). Crone and Lord (1994) observed that organ primordia in floral homeotic mutants of *Arabidopsis* initiate in a normal manner.

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**Fig. 5.** Characterisation of cell death and fourth whorl gene expression in the transgenic, strong phenotype, *Nicotiana*. (A) Light micrograph of longitudinal section. (B) DAPI staining of section in A visualised with fluorescence optics. The cells located between the first and fourth whorls are much brighter than background with fluorescence appearing in the cytoplasm. (C) A similar section to that in A which has been probed with digoxigenin-labelled NAG1 antisense RNA, which shows expression of NAG1 in the retarded fourth whorl. Scale bar, 100 μm.
and only subsequently show deviations in cell number and division patterns from wild type. Therefore, the organ identity genes are probably not required for the initiation of organogenesis. A mechanism establishing organ identity as distinct from that of establishing floral organ position and number can also be inferred from the phenomenon of floral reversion. *Impatiens balsamina* flowers may revert to vegetative growth if the floral stimulus is removed but the number and position of newly developing vegetative primordia remain typical of the floral meristem (Battey and Lyndon, 1984, 1990). Consequently, the development of the organ pattern might be dependent on neighbouring whorls to coordinate the organ positions within the meristem by biochemical (Wardlaw, 1949; Young, 1978) or biophysical signals (Green, 1988). However, our observations suggest that positional coordinates are established in the floral meristem by the stage of sepal primordium initiation.

Although positional coordinates appear to be defined at an early stage of floral development, inductive interactions between cells in different regions of the meristem may occur at an earlier stage. Several genes are expressed in the meristem prior to the initiation of sepal primordia and may have a role in establishing these positional cues. These genes include *floridaula* and *fimbriata* in *Antirrhinum*, and *LEAFY* in *Arabidopsis* (Coen et al., 1990; Simon et al., 1994; Weigel et al., 1992). Mutations in all three of these genes affect the floral phyllotaxy, and so may be required to establish the position of floral organ primordia.

**Inter-whorl signals may cause some abnormalities in *Nicotiana* transgenic flowers**

Fourth whorl development in *Nicotiana* transgenic flowers was retarded, but eventually normal pistils could be observed (Fig. 3B). We suggest that this delay is not due to the loss of an inductive signal, since we observe expression of NAG1 in the fourth whorl region (Fig. 5C). One possibility to explain this retarded growth could be the loss of general growth regulators. Growth regulators such as hormones could be acting to promote carpel outgrowth. Stamens are known to be a source of gibberellins (Weiss et al., 1990; Kinet et al., 1985), and may play a role in regulating the pace of carpel development. In the transgenic *Nicotiana* flowers the loss of such growth regulators may result in the delay and distortion of carpel development. In this scenario, hormones would not be required to specify organ position and number, but would be more general in their action to promote growth. This effect may be more pronounced in *Nicotiana*, due to the length of time required for floral organogenesis in this species. Since no *Nicotiana* mutants affecting stamen development have been described, this hypothesis cannot be tested using mutants. In *Antirrhinum*, a close relative of *Nicotiana*, homeotic mutants *deficiens* and *globosa* result in stamens that are transformed to carpelloid organs (Sommer et al., 1990; Trobner et al., 1992). In these mutants it is not unusual to have a fused third whorl gynoecium with an absent or reduced fourth whorl. It is not clear if this phenotype is due to a similar delay of fourth whorl development or to other factors.

A proportion (56%) of the carpels in the strong transgenic *Nicotiana* phenotype display mosaic patches of petal or stamen tissue. These patches could be due to an inductive signal emanating from the first whorl primordia, which are physically apposed to the fourth whorl cells, such that a signal originally destined to induce second or third whorl development would now act on fourth whorl cells. However, such an inductive signal does not appear to affect all fourth whorl cells, since many of the flowers derived from the strong transgenic line show normal carpel morphology. Such an inductive signal may act late in development, as the fourth whorl cells are differentiating.

An alternative hypothesis to explain the mosaic patches is that some of the second or third whorl cells are not killed by the toxin if the toxicity drops below a threshold. These cells
might be recruited into the fourth whorl forming a mosaic patch of petal or stamen tissue on the side of the gynoecium. The number of cells recruited determines the size of the sector until, in the case of large sectors, there is distortion of the gynoecium (Fig. 3D). This observation suggests that the establishment of the carpel primordia may occur subsequent to cell fate determination in these transgenic *Nicotiana* plants.

**The chimeric diphtheria toxin has a low toxicity**

Diphtheria toxin has been used successfully in several other systems to induce cell death (Bellen et al., 1992; Breitman et al., 1987). Only a few molecules of the toxin are sufficient to kill mammalian cells (Yamaizumi et al., 1978). We have used a modified toxin, with 54 amino acids of the AP3 coding sequence fused to the amino terminal end of the DTA coding region, which appears to have a lower toxicity. This reduced toxicity is apparent in the phenotypic series of the different transgenic *Nicotiana* lines, since the severity of the phenotypes are proportional to copy number. Hence, there seems to be a threshold level of chimeric toxin which must be reached before it is lethal. Since the level of AP3 expression in ovule integuments is relatively low (Jack et al., 1992), threshold levels of toxicity could explain why only some of the integuments are ablated in the AP3-DTA transgenic *Arabidopsis*. Also, mosaic patches of petal and stamen tissue can occasionally be observed on the carpels in the phenotypically strong transgenic *Nicotiana* flowers. A cell which differentiates as a petal or stamen cell in these transgenic flowers should be ablated, but in the mosaic patches the level of toxin produced may not be lethal.

**Pattern formation during floral development**

Our results appear to contradict the conclusions of surgical experiments that inductive interactions between whorls play an important role in establishing the floral pattern until relatively late in development. We propose that the regenerative capability of a floral meristem could be sequentially restricted to individual whorls. In other words, inductive signals within a whorl could function until quite late in development, but inductive interactions between whorls would be restricted to a very early phase of floral development, prior to the expression of AP3. By this model, the results from the surgical experiments could be explained by signals being disseminated within a whorl (Fig. 6). After bisection, only those whorls still competent to transmit such an intra-whorl inductive signal would show regeneration of organs. Since our genetic ablation studies result in complete elimination of individual whorls, no such regeneration is possible. Future experiments, aimed at ablating only part of a whorl, will help to distinguish whether such a model is correct.

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**REFERENCES**


Kulkarni, G. V. and McCulloch, C. A. G. (1994). Serum deprivation induces...
apoptotic cell death in a subset of Balb/c 3T3 fibroblasts. J. Cell Sci. 107, 1169-1179.


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