GENETIC AND MOLECULAR ANALYSES OF THE ROLE OF THE
UNUSUAL FLORAL ORGANS GENE IN REGULATION OF SHOOT AND
ORGAN IDENTITY IN ARABIDOPSIS THALIANA

by

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We accept this as conforming
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ABSTRACT

Shortly after initiation, shoot and floral organ primordia become determined to particular fates based on their spatial/temporal position in the plant. Many genes are known which affect the proper regulation of this process, and these genes are expressed in spatial/temporal patterns consistent with the developmental decisions they regulate. Little is known about the mechanisms by which these regulatory genes achieve their final patterns in the developing plant.

I have identified a novel gene involved in regulation of flower initiation and development in Arabidopsis. This gene is designated UNUSUAL FLORAL ORGANS (UFO). Ufo plants generate more coflorescences than do the wild type and, unlike wild type, coflorescences often appear apical to the first floral shoot, indicating that UFO has a role in regulating coflorescence versus floral fate determination by lateral shoots. In addition, Ufo floral organs exhibit variable homeotic transformations to any of the four organ types in the second, and third whorls, suggesting that ufo mutations result in variable expression levels and/or patterns of the organ identity genes. These hypotheses are supported by phenotypic analysis of double mutants between ufo and other floral initiation and floral organ identity gene mutations. Surprisingly, in situ hybridization analysis revealed no obvious differences in mRNA levels or pattern for any organ identity gene examined. These results suggest that UFO may act post-transcriptionally to regulate the expression of floral organ identity genes.

UFO was cloned by homology to the Antirrhinum gene FIMBRIATA. Sequence
analysis revealed no homology to any other gene in the sequence databases. *UFO* transcription is detected in spatial/temporal patterns consistent with its proposed roles in shoot and organ identity gene regulation, and does not appear to rely directly on any of the floral initiation genes.

Collectively, the data indicate that *UFO* is required as part of the floral initiation process to establish the proper domains of shoot and organ identity gene function, and that this regulation occurs post-transcriptionally.
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ABBREVIATIONS and NOMENCLATURE

aa  amino acid
AG  AGAMOUS
AN  ANGUSTIFOLIA
AP1 APETALA1
AP2 APETALA2
AP3 APETALA3
bp  base pair
°C  degrees Celsius
CAL Cauliflower
CER ECERIFERUM
CL  Continuous light
CLV CLAVATA
Col-2 Columbia-2 ecotype
COPS Control of Phase Switching
DEFA DEFICIENS
df  degrees of freedom
DIG digoxygenin
DIS2 DISTORTED TRICHOMES 2
E einstein
EMF EMBRYONIC FLOWER
ER ERECTA
F1  first filial progeny
F2  second filial progeny
F3  third filial progeny
FAA formaldehyde/acetic acid
FIM FIMBRIATA
FLIP Floral Initiation Process
FLO10 FLORAL MORPHOLOGY10 (aka SUPERMAN)
GLO GLOBOSA
GA4 GIBBERELLIC ACID RESISTANT 4
hr  hour
HY LONG HYPOCOTYL
Kan-r kanamycin-resistant
Kan-s kanamycin-sensitive
kb  kilobase
kV  kilovolts
Ler Landsberg erecta ecotype
LFY LEAFY
m  metre
MADS MCM1, AG, DEFA, SRF
The following rules are accepted as standard rules governing gene/allele nomenclature for *Arabidopsis*: Wild type genes names and symbols should be all upper case and italicized (e.g. *AGAMOUS* or *AG*). Mutant alleles should be all lower case and italicized (e.g. *ag*) and different alleles of the same gene should be distinguished by a hyphen followed by the allele number (e.g. *ag-2*). This nomenclature is used for both dominant and recessive mutations. Phenotypes are designated by the allele symbol with the first letter capitalized in normal type (e.g. *Ag-2*). Gene products are designated by the gene symbol in upper-case normal type (e.g. *AG*).
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1. LITERATURE REVIEW

1.1 Introduction to Arabidopsis morphology

1.1.1 The inflorescence

Mature Arabidopsis plants consist of a basal rosette of closely appressed leaves and an aerial shoot (inflorescence) carrying branches and flowers. In continuous light (CL) conditions four distinct node types are apparent in the mature plant: Juvenile rosette, mature rosette (Medford, et al., 1992), early inflorescence, and late inflorescence. A mature Arabidopsis plant is diagrammed in Figure 1.1. Juvenile rosette, mature rosette, and early inflorescence nodes may be considered vegetative and generate leaves with associated axial shoots. These three node types are distinguished by phyllotaxy of the leaf primordia, the shape of the mature leaf, and/or the degree of internode elongation. Juvenile rosette nodes (approximately the first four nodes; Medford, et al., 1992) generate small rounded leaves at an angle approaching $180^\circ$ from the previous leaf primordium and exhibit no internode elongation. Mature rosette nodes (approximately 7 to 8 nodes) generate increasingly spatulate leaves in a spiral arrangement but still lack internode elongation. Early inflorescence nodes (approximately 2 to 4 nodes) are structurally similar to the late rosette nodes, although they are raised off of the rosette due to internode elongation (bolting). Axial shoots from these nodes generally elongate earlier than those in the rosette, eventually forming a lateral inflorescence (coflorescence) with a morphology
Figure 1.1. Structure of mature *Arabidopsis thaliana* plants grown in CL or SD conditions. Wild type Columbia-2 plants grown under continuous light (left) generate a basal rosette, several coflorescence nodes, followed by a raceme of flowers until plant senescence. Columbia-2 plants grown under SD conditions (right) exhibit an enhanced number of rosette and coflorescence nodes.
similar to the inflorescence from which they were derived. Finally, late inflorescence and coflorescence nodes generate floral shoots with no associated leaf in a racemous arrangement. Flowers are produced until apical senescence. Wild type inflorescences will typically produce 25-40 flowers prior to senescence, although the apical meristem remains competent to generate additional lateral shoots (Hensel, et al., 1994). Thus apical meristems of *Arabidopsis* are considered indeterminate.

The act of bolting is associated with the onset of flowering. It has been proposed that the onset of floral initiation causes a 'signal' to be sent from the shoot apex towards the base of the plant that leads to internode elongation (Hempel and Feldman, 1994). In this hypothesis, internode elongation between early inflorescence nodes is the result of floral initiation rather than a change in node type between mature rosette and early inflorescence. This is supported by the observation that internode elongation between late rosette nodes may occur late in plant development, apparently increasing the number of coflorescence nodes (data not shown). Thus it is possible that only two growth phase switches occur in *Arabidopsis*: from juvenile to mature rosette, and from mature rosette to flowering, where the act of flowering causes the most apical rosette nodes to elongate off of the rosette and their associated coflorescences to develop further.

1.1.2 The flower

*Arabidopsis* flowers consist of four types of organs in a consistent spatial arrangement (Figure 1.2). An outer whorl of four green sepals make up the first whorl.
Figure 1.2. Structure of a mature Arabidopsis flower. Diagram shows the arrangement of the four whorls of organs. The outer two whorls are termed 'perianth' whorls, while the inner two are 'reproductive' whorls.
Interior and alternate to this is a whorl containing four white petals. These two whorls make up the 'perianth' whorls. The third whorl contains six stamens; two short stamens and four long stamens. Two opposing carpels are produced in the fourth whorl, which are fused to form the pistil. Whorls three and four make up the 'reproductive' whorls. The floral apical meristem is not conspicuous after production of fourth whorl organs and does not develop further under normal conditions, thus the apical meristem of an Arabidopsis flower, unlike the inflorescence apex, is considered determinate.

Maintenance of indeterminacy in inflorescence apices has been studied in some detail. It has been proposed that the indeterminacy of an inflorescence meristem is dependant on the production of flowers by that meristem and, more specifically, on the fertilization of those flowers. In male sterile plants, and plants in which the flowers have been surgically removed, inflorescence meristems proliferate beyond that expected from wild type, and finally terminate with the production of flower-like structures (particularly carpels; Hensel, et al., 1994). However, it is possible that under normal conditions all Arabidopsis inflorescence meristems are determinate but senesce before termination is observed. Thus developing fruits may regulate senescence rather than determinacy. Regardless, it is apparent from these data that some mechanism must exist to allow development of the plant body through maintaining the indeterminate nature of the inflorescence meristem and, under certain circumstances, this mechanism fails or is no longer active, causing the inflorescence apex to adopt the fate of a floral apex.
1.1.3 Effect of environment on growth of *Arabidopsis*

Time to flowering is controlled by both genetic and environmental factors, including day-length and temperature. Flowering plants can be generally divided into three categories based on their photoperiodic responses: 1) short-day (SD) plants require extensive, uninterrupted periods of dark, and brief periods of light in order to flower, 2) long-day (LD) plants require brief periods of dark and extensive periods of light to induce flowering, and 3) day-neutral plants, which require no specific day/night regime to induce flowering. In addition to these broad classes, many plants, such as *Arabidopsis*, show a quantitative response to the day/night regime; *Arabidopsis* plants flower earlier in LD, but will eventually form flowers in SD conditions. As such, *Arabidopsis* is considered a facultative long-day plant.

In continuous light wild type *Arabidopsis* plants generate approximately nine rosette leaves prior to bolting. In short days (SD) the number of rosette nodes may increase to greater than 60 prior to the onset of flowering. Secondary and tertiary coflorescence number are also increased, however floral morphology is not affected by day length.

The remainder of this work will describe mutations affecting floral and inflorescence morphology. In many cases the structures described will not fit the terminology used to describe wild type structures, thus it is necessary to define terms that will unambiguously describe a structure or position on a plant. Throughout this work meristems will be described in the following manner: The meristem of the primary (1⁰) shoot will be defined as the 1⁰ apical meristem, or 1⁰ apex. Meristems of coflorescence
shoots will be defined according to the degree to which they are separated from the primary shoot. Coflorescences emerging from the 1° shoot will be defined as secondary (2°) shoots with 2° apical meristems, coflorescences from 2° shoots will be defined as tertiary (3°) shoots, and so on. Secondary shoots that are flower-like in morphology and exhibit no 3° lateral branching will be described as flowers, however, in many cases lateral structures can not be accurately described as either coflorescence or flower. In such situations the node type will be defined according to what type of structure is produced in wild type plants at that position. Thus, ambiguous structures generated in the position expected of a flower (based on wild type morphology) will be defined as floral shoots regardless of the morphology of the structure generated. 'Determinate' will be used to describe meristems that appear to form a terminating structure prior to senescence of that meristem. 'Indeterminate' will be used to describe cases where the apical meristem remains clearly visible at the point of senescence.

Since developmental programs tend to interact extensively with one another it is difficult to discuss any single developmental phenomenon in isolation. The final phenotype is the combination of what did not happen with what happened instead; the alteration or removal of one program usually results in a 'compensatory' response by a different program. Further, it is becoming clear that the divisions we make between different developmental processes, and the assignment of genes to these processes, are largely artificial. Thus it will be necessary at times to refer the reader to other sections of this manuscript to explain the causes of certain mutant phenotypes.
1.2 Regulation of phase switching (COPS)

As previously described, Arabidopsis plants pass through several developmental phases prior to apical senescence. The duration of each phase is largely consistent from one plant to another, and each phase is comparably affected by environmental factors such as cold treatment (vernalization) and photoperiod (day-length). Vernalization decreases the number of nodes produced before flowering, and can be mimicked by treatment of seeds with gibberellins (see Burn et al., 1993). Similarly, long days also decrease the number of nodes in each developmental phase. In addition to environmental factors, several mutations have been identified that affect the duration of each phase, while still allowing all phases to occur. These data together suggest that a 'control of phase switching' (COPS) mechanism exists that regulates the transition from one phase to another, and that this mechanism is controlled both genetically and environmentally (Haughn et al., 1995; Schultz and Haughn, 1993).

1.2.1 COPS mutant phenotypes

Mutations in COPS genes affect all developmental phases in the same manner and are therefore distinct from mutations in genes that regulate only one developmental phase (for example floral initiation). COPS mutations fall into two main phenotypic classes: early flowering and late flowering. Although many early and late flowering mutations have been identified only two mutants will be discussed in any detail here (for a more
complete review see Haughn, et al., 1995). Mutations in the early flowering genes
*TERMINAL FLOWER (TFL)* (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992,
Zagotta, et al., 1992) and *EMBRYONIC FLOWER (EMF)* (Sung et al., 1992) result in
plants with reduced numbers of rosette and coflorescence nodes. In addition, premature
termination of apical growth occurs, associated with the production of flowers or flower-
specific organs, particularly carpels, at the shoot apex. Apical termination of Tfl and Emf
mutants makes them distinct from other early flowering mutants in which all inflorescences
remain indeterminate. These mutations are believed to represent 'loss of function' alleles,
thus this phenotype suggests that *TFL/EMF* activities are required to promote vegetative
fate determination by lateral shoots, and suppress flower-specific developmental programs
in the apical meristem; *TFL/EMF* activity in any meristem is antagonistic to floral fate in
that meristem (Alvarez et al., 1992; Shannon and Meeks-Wagner, 1991; Shannon and
Meeks-Wagner, 1993; Schultz and Haughn, 1993). The Emf phenotype is stronger than
that of Tfl, and some 'strong' *emf* alleles cause carpel production and apical termination
immediately after germinating.

Tfl and Emf differ in their response to photoperiod. As in wild type, SD increases
the number of rosette and coflorescence nodes in Tfl plants (Shannon and Meeks-Wagner,
1991), consistent with the designation of *TFL* as a regulator of phase switching rather than
a phase-specific gene. However, termination of Tfl inflorescence apices still occurs under
SD. Emf plants are not responsive to daylength and, even in SD, strong *emf* alleles
produce only carpels from the 1° apex (Sung et al., 1992). Thus it is possible that Emf
represents a complete loss of COPS activity, while in Tfl COPS is weakened but active.
1.2.2 Model of COPS activity in *Arabidopsis*

Based on these data, it has been proposed that these two genes, particularly Emf, generate the COPS 'signal', while other late and early flowering genes respond to environmental stimuli and modulate the level of this signal accordingly (Schultz and Haughn, 1993, Haughn, et al., 1995). Thus, in short days, COPS activity is increased, resulting in a longer phase of vegetative growth. Similarly, vernalization and long days decreases the COPS signal causing the plant to flower earlier.

Figure 1.3 shows a model that explains how COPS may produce the wild type and mutant phenotypes described. In this model, COPS activity (or the plants’ response to COPS activity) decreases apically, allowing a switching of developmental programs as COPS passes through various activity thresholds. Under SD, COPS must decrease more slowly, causing each phase to contain more nodes. In wild type plants, senescence likely occurs before COPS activity is low enough to allow the inflorescence apex to undergo floral differentiation/termination, thus inflorescences appear indeterminate. In Tfl mutants, COPS activity is reduced, such that each threshold is reached at an earlier node, and COPS activity becomes sufficiently low in the apex that differentiation of the apical meristem occurs. In Emf, little or no COPS activity is present in the plant, resulting in immediate apical differentiation into carpels.
Figure 1.3. Proposed explanation for COPS regulation of phase switching in wild type and mutant Arabidopsis plants.
Figure 1.3: Proposed explanation for COPS regulation of phase switching in wild type and mutant *Arabidopsis* plants. Four cases are shown which are representative of plants with increasing amounts of COPS activity from left to right. The width of the vertical triangular gradients indicate the amount of COPS activity, decreasing acropetally in the plant. Dotted lines show the position at which the plant reaches each COPS threshold and switches to the subsequent developmental phase. Emf plants are believed to have little or no COPS activity. Tfl plants have reduced COPS activity. Wild type Col-2 plants in CL have less COPS activity than wild type Col-2 plants grown in SD.
These observations lead to a novel view of plant development in which all plant structures result from an antagonistic battle between vegetative programs (controlled by COPS) and the carpel program (controlled by AGAMOUS (AG) - discussed in section 1.4) (Haughn et al., 1995). In the absence of COPS activity, meristems assume the carpel fate. Other developmental programmes, such as those controlling development of floral organs, have been overlayed on these two fundamental processes. High levels of COPS activity promote vegetative developmental programmes and inhibit AG, intermediate levels of COPS activity promote flower-specific genes that in turn inhibit AG, while at very low levels of COPS, AG activity is allowed and carpel development is initiated. This hypothesis is supported by a diverse set of observations, and will be discussed further when appropriate throughout this work.

1.3 Regulation of flower initiation (FLIP)

It should be noted that regulation of floral initiation, and the subsequent regulation of floral development, are overlapping processes that share several genes. Thus, although these two processes are separated here for convenience, it is becoming apparent that this separation does not properly reflect the biology of the system and many of the genes described will be discussed further in the subsequent section.

One of the developmental phases controlled by COPS is the vegetative to floral transition, which occurs between early inflorescence and late inflorescence nodes (Figure 1.1). This transition is associated with the activation of a group of genes termed the 'floral
initiation process' (FLIP) genes in the cells of the first 2° shoot that is to become a flower and each subsequent 2° shoot primordium. Four of these genes, LEAFY (LFY), APETALAI (AP1), CAULIFLOWER (CAL), and APETALA2 (AP2) have been studied in detail. Mutations in any of these genes result in complete or partial transformation of flower to coflorescence, indicating that mutant plants are unable to properly initiate the floral developmental program; however coflorescence features become less dramatic acropetally and the lateral shoots become gradually more flowerlike. The inflorescence structure of each mutant is diagrammed in Figure 1.4.

Leafy mutants exhibit the strongest transformation of flower to coflorescence. Lfy 'floral' shoots have elongated internodes with leaves and 3° axial shoots at each node, and only rarely are sepals, petals or stamens produced. These leaves become more carpelloid in apical nodes and all Lfy inflorescences and coflorescences terminate with the production of carpel-like organs (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992). This feature suggests, once again, that the carpel developmental program is independant of the rest of the floral program and antagonistic to vegetative development. The degree of lateral shoot formation and internode elongation decreases in apical nodes, making these shoots more 'flowerlike', although floral organs other than carpels are rare. Growth of Lfy plants in SD enhances the severity of flower to coflorescence transformation (Schultz and Haughn, 1993).
Figure 1.4. Morphology of wild type and FLIP mutants under continuous light conditions.
Figure 1.4: Inflorescence structure of FLIP mutants. Wild type morphology is shown (left) for comparison. Lfy-1, Ap1-1, and Ap2-1 exhibit complete or partial transformation of flower to coflorescence. Note that the severity of the mutant phenotype decreases from left to right, with Lfy showing the strongest transformations, Ap1 being intermediate, and Ap2 showing weak transformations.
Apetalal mutants (Irish and Sussex, 1990; Huijser et al., 1992; Mandel, et al., 1992; Bowman et al., 1993; Schultz and Haughn, 1993) exhibit complete and partial flower to coflorescence transformations, which become less pronounced acropetally. In strong alleles, basal flowers are often completely transformed to indeterminate, coflorescence-like structures. More apical 'floral' shoots are determinate, in that they eventually produce flower-like structures at the shoot apex, however the base of the shoot exhibits a developmental pattern similar to that of a coflorescence, having internode elongation with 3° shoots at each node. Flower-like structures from the apices of these shoots consist of a first 'whorl' of leaves and axial shoots with slight internode elongation between the lateral and medial pairs of first whorl organs. Few or no second whorl organs are produced, but a relatively normal third and fourth whorl, both in organ type and number, is generated. If second whorl organs are present they usually exhibit partial or complete transformation to stamen. 3° shoots from the basal regions of these unusual flowers may be indeterminate; alternatively, they may follow a developmental pattern similar to that of the 'flower' from which they were derived, generating 4° shoots with elongated internodes followed by a normal third and fourth whorl. Growth of Ap1 plants in low temperatures, or SD, enhances vegetative-like growth of lateral shoots (Schultz and Haughn, 1993). This phenotype suggests that, like LFY, API is required for proper initiation of the floral program and that, in its absence, coflorescence and floral developmental programs are mixed. Weaker ap1 alleles produce second and third whorl organ types consistent with varying levels of AG activity in these whorls (see subsequent section on regulation of floral organ type) indicating that API is also involved in regulating
Mutations in the *CAL* gene have no conspicuous phenotype alone, but enhance the phenotype of *ap1* mutations (Bowman et al., 1993). Every shoot in floral position is transformed into an inflorescence-like meristem that generates lateral shoots in a spiral phyllotaxy with little or no internode elongation. These 3° meristems also act as inflorescence-like meristems, following a developmental pattern similar to the shoot from which they were derived. This pattern reiterates several times, resulting in a shoot that resembles a small cauliflower. The last meristems to be formed occasionally differentiate into flowers with a phenotype similar to that of Ap1 (described above). This phenotype exhibits an acropetal decrease in severity (Bowman et al., 1993).

Mutations in the *APETALA2* exhibit the weakest flower to coflorescence transformations of the known FLIP genes. Alleles of *ap2* fall into two phenotypic classes: Those that exhibit transformation of perianth organs to carpels and stamens (e.g. *ap2-6* - Kunst et al., 1989), and those in which the outer whorl is absent and replaced by several nodes producing lateral shoots (e.g. *ap2-1* - Bowman et al., 1989). It is the latter of these phenotypes that allows *AP2* to be included as a member of the FLIP gene class, and Ap2-1 plants often look similar to certain alleles of *ap1*, particularly when grown in SD (Schultz and Haughn, 1993).

Mutations in pairs of FLIP genes have a synergistic effect on flower to coflorescence transformations (Haughn, et al., 1995; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). This suggests that the FLIP genes act together to initiate the floral program; however, the increasingly 'floral' phenotype of
apical shoots from FLIP mutants suggests that the floral program is not entirely dependant on the activity of any one FLIP gene (Haughn et al., 1995; Schultz and Haughn, 1993; Bowman et al., 1993). It has been suggested that floral developmental programs may be inhibited directly by COPS activity and that, as COPS activity decreases, individual floral programs become de-repressed even when FLIP activity is low. Thus, in addition to coordinately activating the floral programs, FLIP genes may also be required to reduce COPS activity in a shoot primordium that is destined to become a flower (Schultz and Haughn, 1995). These interactions are diagrammed in Figure 1.5. When the FLIP 'decision' is made, this decision is reinforced through positive interactions among the FLIP genes, and negative interactions between the FLIP genes and COPS, resulting in a dramatic reduction of COPS activity and a clear distinction between coflorescence and floral nodes.

1.4 Regulation of floral organ identity

Regulation of floral organ type is the most studied developmental processes in Arabidopsis. Figure 1.6 shows a simple model for the regulation of floral organ identity in Arabidopsis flowers, which has been derived through genetic and molecular analysis of mutations in a number of genes affecting organ type (Haughn and Sommerville, 1988; Kunst et al., 1989; Bowman et al., 1991). In this model, specification of organ type is achieved through the actions of three sets of genes, named class A, B and C, which are expressed in unique but overlapping domains within the developing flower. Class A activity is present in whorls 1 and 2, class B activity is present in whorls 2 and 3, and class
Figure 1.5. Regulatory interactions between the COPS mechanism and the FLIP.
Figure 1.5: Regulatory interactions between the COPS mechanism and the FLIP. The vertical triangular gradient represents the acropetal decrease in COPS activity in the plant. The horizontal dashed line represents the threshold COPS level at which FLIP genes are activated. Arrowed lines represent positive regulatory interactions. Barred lines represent inhibitory interactions.
C activity is present in whorls 3 and 4. Thus, each organ primordium is specified through a unique combination of organ identity gene activities. Sepals are specified by class A alone, petals by class A+B, stamens by class B + C, and carpels by class C alone. Overall, class A specifies perianth structures, class C specifies reproductive structures, and class B distinguishes which perianth, or reproductive structure is produced. This model suggests that organ primordia do not become determined on the basis of their position within the flower, but according to the combination of organ identity gene activities present in each primordium. Thus it is the spatial patterning of the organ identity gene activities, overlayed with the correct spatial patterning of organ primordia, that accounts for the final pattern of floral organs. If either of these become disrupted inappropriate organ types will be produced.

Other features of this model, including interactions among organ identity genes and between organ identity genes and FLIP genes, become apparent through discussion of the mutant phenotypes upon which the model was built. Figure 1.7 diagrams the final patterns of organ identity gene activity in various classes of organ identity gene mutants, while Figure 1.8 diagrams the proposed interactions between organ identity genes (discussed in later sections of this document).
Figure 1.6: Model for genetic control of wild type *Arabidopsis* flower development. Diagrammatic view of a longitudinal section through a wild type flower. Whorls are numbered 1–4. Domains of class A, B, and C gene activity are indicated by angular boxes, with the gradients representing changes in the strength of activity for each gene class. Each box is labelled with known representative genes. The position of *FLO10* gene activity is also indicated.
1.4.1 Class A activities

There is significant overlap between class A organ identity gene members and FLIP gene members. As described earlier, certain FLIP mutations such as \textit{ap1-1} and \textit{ap2-1} result in loss of perianth whorls, and the development of several coflorescence-like nodes in their place. Other alleles of \textit{AP2}, and to a lesser degree \textit{API}, affect primarily organ identity in the first two whorls (Kunst et al., 1989; Bowman et al., 1989). Thus these two genes also play a role in the specification of perianth organ type. Figure 1.9 shows the relative role of each FLIP gene in the various flower-specific processes that they regulate (Wilkinson and Haughn, 1995; Haughn et al., 1995; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993; Weigel and Meyerowitz, 1993). The vertical axis represents their relative strength in making the floral initiation decision, while the horizontal axis represents a breakdown of the flower-specific processes that are regulated by FLIP genes. It is apparent that \textit{LEAFY} regulates most floral functions while \textit{AP1} and \textit{AP2} regulate a subset of these functions, including specification of perianth organ types (class A activity).

In Ap2-6 outer whorl organs develop as carpels while second whorl organs also have a carpel-like appearance (Kunst et al., 1989). Similarly, certain alleles of \textit{ap1} also exhibit partial petal to stamen transformations (Schultz and Haughn, 1993; Bowman et al., 1993). The replacement of perianth organs with reproductive organs indicates that class C activity occupies the class A domain when class A activity is absent, thus class A and C activities are mutually antagonistic (Fig 1.7B).
Figure 1.7: Final pattern of floral homeotic gene activity in four classes of floral mutants.
Figure 1.7: Final pattern of floral homeotic gene activity in four classes of floral mutants. Domains of activity of the three organ identity gene classes are represented in wild type (A), class A mutants (B), class C mutants (C), class B mutants (D), and Flo10 mutants (E). Whorls are numbered 1-4.
Figure 1.8: Interactions between the organ identity genes. Exploded view of organ identity gene patterns showing genetic and/or molecular interactions between each gene class. Arrowed lines indicate positive regulatory interactions, while barred lines represent negative regulatory interactions.
The *ap2-I* allele is an atypical in that only one of the roles of a class A gene has been affected. Ap2-1 flowers generate leaves and lateral shoots in the first whorl, and petal-stamen mosaic organs in the second whorl. It is believed that this results from the loss of class A organ identity activity, but the retention of class C antagonism. Thus in the first floral whorl no organ identity gene activity is present, resulting in leaves and flowers, while in the second whorl class B activity alone is active (Bowman et al., 1989, 1991, 1993). This phenotype supports the hypothesis that the floral program is antagonistic to COPS since vegetative growth enters the flower when class A activity is absent. In addition, second whorl petal-stamen organs indicate that class B activity alone is able to initiate both petal and stamen development, and it is the role of class A or C genes to determine which of these two fates prevail in a given organ primordium.

In addition to their effects on perianth organ type, certain class A gene mutants also affect third and fourth whorl organs to a lesser degree. In Ap2-6, fewer third whorl organs are produced and within the fourth whorl, ovule development is altered (Modrusan et al., 1994). Reduction in third whorl organ number is consistent with the proposal that *AG* activity influences determinacy; the production of carpels due to class C activity in the first two whorls may decrease the overall proliferation of the floral meristem. In addition there is some evidence to suggest that *AG* activity has a negative effect on class B function (Schultz et al., 1991). The role of Ap2 in ovule development has also been extensively studied (Modrusan et al., 1994).
Figure 1.9: Role of FLIP genes in flower-specific developmental processes. The vertical gradient (left) represents an axis to compare relative participation of LFY, AP1/CAL, and AP2 in the FLIP. Each box spans a range of flower-specific activities (bottom) in which that FLIP gene participates.
Recently the role of the *LEUNIG (LUG)* gene was described (Lui and Meyerowitz, 1995). Lug mutants exhibit ectopic expression of *AG* similar to Ap2 flowers, although it was shown that *LUG* has no organ-identity activity of its own. It has therefore been proposed that *LUG* is primarily a regulator of class C activity, keeping *AG* out of the perianth whorls.

1.4.2 Class C activities

Mutations in the only known class C gene, *AGAMOUS*, result in a normal first and second whorl of sepals and petals respectively, followed by transformation of third whorl organs to petals, and the replacement of the fourth whorl with what is apparently a new floral primordium. From this 'nested' primordium, the pattern of (sepal, petal, petal)$_n$ reiterates, with internode elongation between each nested flower (Yanofsky et al., 1990; Bowman et al., 1991). This phenotype indicates several functions for class C genes beyond specification of reproductive organ type. The appearance of petals in the third whorl indicates that class A activity is present in the third whorl of Ag flowers, thus *AG* appears to exclude class A activity from the reproductive whorls (Fig. 1.7C) Further, the reiteration of the floral program indicates that *AG*, or at least the carpel program itself, is somehow involved in specifying floral determinacy. In the absence of *AG*, the flower becomes an indeterminate structure (Yanofsky et al., 1990; Bowman et al., 1991). This is consistent with the indications from COPS and FLIP mutants that apical indeterminacy is lost following the production of carpels at the apex.
1.4.3 Class B activities

Class B mutants such as *pistillata (pi)* and *apetala3 (ap3)* generate sepals in the first and second whorl, and carpels or filamentous structures in the third and fourth whorls. Third and fourth whorl carpels frequently fuse to form a multicarpellate gynoecial structure (Bowman et al., 1989, 1991; Hill and Lord, 1989; Jack et al., 1992).

Regulation of class B activity is achieved through the action of the *FLOW* gene (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 1995). Flo10 mutants generate normal first, second, and third whorl organs, however carpels are replaced by several whorls of stamens before the flower finally terminates with the production of stamen-carpel mosaic structures. Mutations in class B genes are epistatic to these stamen transformations indicating that the role of *FLO10* is to exclude class B activity from the fourth whorl (Fig. 1.7D, 1.7E). Again, the loss of carpels in Flo10 flowers is associated with loss of floral meristem determinacy.

1.5 Studies of floral initiation and development in other systems

Much of the work done on flower initiation and development using the *Arabidopsis* system has been done in parallel with work done in other plant systems, in particular *Antirrhinum majus*. Homologs of most of the *Arabidopsis* genes discussed in the previous sections were either initially identified in *Antirrhinum*, or have since been isolated in that system. *Antirrhinum* class A and FLIP genes include *FLORICAULA (LFY)*, *SQUAMOSA (AP1)*, and *SQUAMATA*. The class B genes *DEFICIENS (AP3)* and *GLOBOSA (PI)*, as
well as the class C gene *PLENNA (AG)* have also been identified (for a recent review see Davies and Schwarz-Sommer, 1994). All genes exhibit strong identity in both phenotype and sequence to the *Arabidopsis* homologs, and a similar model for regulation of flower development have been suggested (Schwarz-Sommer et al., 1990).

The models derived from studies in *Arabidopsis* and *Antirrhinum* are likely to be representative of the mechanisms used to regulate floral initiation and organ identity in a wide variety of plant species. Mutant phenotypes similar to those observed in *Arabidopsis* and *Antirrhinum* have been described in a wide variety of plant species (Meyerowitz et al., 1989). For example, putative and/or confirmed homologs of AG have been identified and cloned from both monocot (*Zea* maize - *ZAG*), and dicot species (eg. *Tomato* - *TAG*; Puneli et al., 1994), and putative homologs of *AG*, *AP3*, *PI*, and *API* have been identified in the dioecious flowers of White Campion, based on sequence similarity and *in situ* transcript patterns (Hardenack et al., 1994). Similarly, the FLIP mechanism also appears to be conserved through a wide array of plant species. For example, a putative *LFY* homolog has been identified in *Nicotiana* (Kelly et al, 1995), and it was recently reported that the *Arabidopsis* *LFY* gene was sufficient to induce reproductive growth in transgenic Aspen (Weigel and Nilsson, 1995), indicating that both the floral initiation mechanism, and the genes themselves, are functionally conserved in these diverse plant species.

1.6 Molecular nature of the FLIP and organ identity genes

In the past 5 years many of the known FLIP and all of the known organ identity genes have been cloned through insertional tagging, use of heterologous probes (primarily
from Antirrhinum majus), or by screening libraries using specific conserved domains within previously cloned Arabidopsis genes. Among these, AP1, CAL, AP3, PI, and AG all share a common domain that has nucleotide and amino acid sequence similarities with regions of previously cloned loci coding for Mini-Chromosome Maintenance (MCM1 - yeast; Passmore, et al., 1988), Serum Response Factor (SRF - mammal; Norman, et al., 1988), and DEFICIENS (DEFA - Antirrhinum; Schwarz-Sommer et al., 1990; Sommer et al., 1990). This region of similarity has been termed the MADS domain (MCM1, AG, DEFRA, SRF). MCM1 and SRF are transcription factors, and the MADS domain in these loci is known to be involved in DNA binding and dimerization. Thus it is likely that the Arabidopsis MADS genes represent transcriptional regulators (Jack et al., 1994; Goto and Meyerowitz; 1994; Huang et al., 1993; Shiraishi et al., 1993; Schwarz-Sommer et al., 1992; Trobner et al., 1992). AP2 was cloned through insertion of the Agrobacterium T-DNA sequence. It shares no sequence similarity to any known eukaryotic gene, however the amino acid sequence shows certain features common to proteins involved in transcription initiation, thus AP2 may also be a transcription factor (Jofuku et al., 1994).

The functional redundancy between AP1 and CAL suggested that these genes might encode proteins with similar functions. Using AP1 as a probe, a new MADS gene (CAL) was isolated that had 76% overall similarity to AP1 and had a conserved amino acid sequence at 55 of 56 amino acid residues in the MADS domain. This sequence complemented the extreme phenotype of Ap1 Cal double mutants, including a suppression of some aspects of the Ap1 phenotype alone, indicating that Cal was sufficient to substitute for some Ap1 functions. In situ hybridization shows that CAL RNA is present at low levels in a spatial pattern similar to that of AP1, consistent with its apparent functional
With the exception of AP2, all cloned genes have shown in situ transcript patterns consistent with the domains affected by their respective mutations (described later). Although Ap2 mutants are affected primarily in the first and second floral whorls, AP2 transcript is reported to be present not only in the first two floral whorls, but throughout the plant (Jofuku et al., 1994). It is not clear what role AP2 has in third and fourth whorl development, however AP2 is known to be required for proper ovule development (Modrusan et al., 1994)

Certain predictions made in the model for flower development in Figures 1.6 and 1.7 have been supported by molecular analysis of these genes. The proposed antagonistic interactions between class A and C organ identity genes are supported by in situ hybridization studies that have shown that AG transcripts are present throughout flowers from Ap2-2 mutants (Drews et al., 1991) and conversely, API transcript is present in all whorls of Ag flowers (Mandel, 1992), though the API pattern is normal in Ag plants.

Evidence is available that the organ identity genes are activated by FLIP genes. Transcript patterns of the class B genes AP3 and PI are reduced in Lfy mutants, and are completely absent from Lfy Ap1 double mutants, indicating that these genes depend on FLIP function for their activation (Jack et al., 1994; Weigel and Meyerowitz, 1993; Weigel et al., 1992; Huala and Sussex, 1992; Schultz and Haughn, 1991). Maintenance of class B transcription, however, appears to require additional interactions. Products of the Antirrhinum genes DEFA and GLOBOSA (GLO), the homologs of AP3 and PI respectively, have been shown to interact in order to up-regulate their own transcription (Zachgo, S. et al., 1995; Schwarz-Sommer et al., 1992; Trobner et al., 1992). This appears to also be
true for \textit{AP3} and \textit{PI} (Goto and Meyerowitz, 1994) and is supported by \textit{in situ} hybridization studies showing that \textit{PI} transcripts are found in early stages of Ap3 flower development but diminish in later stages while, conversely, \textit{AP3} transcript levels also diminish in later stages of Pi flower development. This accounts for both the identical phenotypes of Ap3 and Pi mutants, and their differing early transcript patterns; \textit{PI} transcript is initially detectable in fourth whorl tissue, however \textit{AP3} transcript is absent. Since they are unable to interact \textit{PI} is quickly lost from the fourth whorl, and carpel rather than stamen development occurs (Goto and Meyerowitz, 1994; Jack et al, 1994).

Negative interactions between class C and class B genes have also been observed by \textit{in situ} hybridization studies. In Ap2-2 mutants, where \textit{AG} activity is present in all floral whorls, both \textit{AP3} and \textit{PI} transcript levels are reduced (Bowman et al, 1991).

The \textit{FLO10} locus has recently been cloned, and has been reported to be transcribed in the interface between third and fourth whorl organ primordia (Sakai et al., 1995). This has led to two proposals for \textit{FLO10} function: 1) that \textit{FLO10} is a regulator of cell division, preventing class B-expressing cells from entering the fourth whorl, or 2) that \textit{FLO10} is a regulator of class B gene product diffusion between third and fourth whorl cells. To explain the Flo10 phenotype it is only necessary to account for regulation of \textit{AP3} activity in the fourth whorl, as \textit{PI} is normally transcribed in early fourth whorl cells and thus is available for the autoregulatory activities of class B genes.

1.7 Transgenic phenotypes using FLIP and organ identity gene constructs

Cloning of the FLIP and organ identity genes allowed generation of plants in which
these genes were transformed into the wild type genome through Agrobacterium mediated gene transfer. To date, LFY, API, AP3, and AG have been analysed as transgenic constructs.

Transgenic plants in which the LEAFY coding region was put under the control of the Tobacco Mosaic Virus 35S promoter (35S) exhibited conversion of all lateral coflorescences into flowers, and premature termination of apical growth associated with the appearance of a flower-like structure from the primary apical meristem (Weigel and Nilsson, 1995). This phenotype was expected given the prediction, from the Lfy mutant phenotype, that LEAFY is required for floral initiation. Surprisingly, rosette leaf number was unaffected, indicating that the apical meristem is not competent to respond to LFY activity until after the rosette phase; thus, this phenotype is distinct from that of Terminal Flower. The transgenic phenotype was significantly, through not completely, suppressed in an ap1//ap1 mutant background. This indicates that API function is largely responsible for the coflorescence to flower transformations seen in the 35S: LFY transgenic plant (Weigel and Nilsson, 1995).

A similar phenotype is seen in transgenic plants carrying a 35S::API construct. All inflorescence shoots are converted to flower-like structures, including the primary apex. This is surprising since loss of API function results in only partial conversion of flowers into coflorescences (Mandel and Yanofsky, 1995). Further, since flowers in the transgenic plant are essentially wild type, this results shows that API alone is not sufficient for regulation of AG, nor for conferring class A activity on organ primordia. The same 35S:API construct in a Lfy mutant causes a similar transformation of inflorescences into flower-like structures; however these flowers show a range of phenotypes typical of those
seen in weaker Lfy mutations, consisting solely of sepals and carpels. These results indicate that *AP1* alone is sufficient for floral initiation, but requires *LFY* activity to correctly initiate downstream pathways, consistent with the 35S::*LFY;ap1//ap1* phenotype described above.

Transgenic plants carrying 35S::*AP3* exhibit transformation of carpel to stamen (Jack et al., 1994). This is consistent with the function of *AP3* as a class B gene. Both *AP3* and *PI* are believed to be necessary for class B function, and the transgenic phenotype was completely suppressed in a 35S::*pi//pi* background. This suggests that *PI* activity is also established in the fourth whorl of transgenic flowers, and was confirmed by *in situ* hybridization to *PI* transcript. *AP3* protein is present in the second, third and fourth whorls of transgenic flowers, however is absent from first whorl organs, and other plant tissues. This indicates that *PI* activity regulates *AP3* post-transcriptionally (Jack et al., 1994).

Consistent with the proposed class C function of *AGAMOUS*, 35S::*AG* transgenic plants exhibit a range of phenotypes similar to those of Ap2 mutant plants. This supports the hypothesis that *AG* negatively regulates class A activity (Mizukami and Ma, 1992).

1.8 Overview of flower initiation and development

Upon seed germination a 'timing' mechanism, termed COPS, is activated. The mechanism by which COPS measures time is not clear; it could be envisioned as a gradient which activates specific sets of genes at threshold levels, however the term 'COPS' is used to describe the phenomenon rather than to imply a mechanism. COPS activity is dependant
on both genetic and environmental factors. At certain levels of COPS activity the fate of lateral shoots changes from early to late vegetative, and from late vegetative to floral.

The initiation of flowers is associated with activation of a set of genes termed FLIP genes. Each FLIP gene is activated independently, but all FLIP genes mutually up-regulate the other’s activities. FLIP activity is antagonistic to COPS activity. FLIP genes have two roles: (1) FLIP genes are initially transcribed throughout the floral meristem, and are required to activate several floral-specific programs such as whorled versus spiral phyllotaxy, suppression of lateral shoot growth, and some floral organ identity functions. (2) Subsequent to floral initiation, FLIP genes take on various patterns of transcription, and appear to be required for regulation of organ identity in the perianth whorls; this is referred to as class A activity.

Specification of the four floral organs is achieved through three overlapping sets of activities. Class B activity is required to specify petal and stamen fates. Class A activity is required to specify sepals in whorl 1 and, in combination with class B, to specify the petals in whorl 2. Class C is required alone to specify carpels in whorl 4, in combination with class B to specify stamens in whorl 3, and is also required to confer determinacy to the floral meristem.

Spatial regulation of floral organ identity genes is achieved in several ways. Class A and C activities are mutually antagonistic, although it is not clear how the second/third whorl boundary between these activities is specified. It is likely that the product of the LUG gene is involved, although no molecular information is available for LUG. Class B genes are strongly dependent on FLIP activity for their activation, and maintain their activity through autoregulation. Autoregulation occurs only when both class B genes are
active, thus class B activity is limited to the second and third whorls. The third/fourth
whorl boundary of class B activity is dependant on the activity of \textit{FLO10} although little is
known about the regulation of \textit{FLO10} itself. Nothing is known about the regulation of the
first/second whorl boundary of class B activity.

1.8 Thesis objectives

The mechanisms through which organ identities are regulated are not fully
understood. In particular, almost no information is available regarding the spatial
regulation of organ identity activities. It is clear that FLIP genes play an important role in
activating these processes, and that the final patterns are maintained largely through
antagonistic interactions both among the organ identity genes, and between these genes and
their regulators; however it is not clear how the initial patterns of expression of organ
identity genes or their regulators are established. In particular, the boundary between class
A and C organ identity genes is apparently maintained through antagonistic interactions
between these two gene classes, however class A genes are activated prior to class C
genes, thus a mechanism must exist to establish this boundary. Similarly, regulation of the
inner boundary of class B activity appears to depend on \textit{FLO10} activity in cells which lie
on this boundary; however it is not clear how the \textit{FLO10} domain itself is established, nor
are any regulators known which establish or maintain the outer boundary of class B
activity.

In this thesis I have used a molecular-genetic approach to investigate the regulation
of floral initiation and floral organ identity. I first describe a mutant phenotype, termed
'Unusual floral organs' (Ufo) that appears to result from disruptions in the normal patterning of organ identity activities, indicating that the affected gene is required for spatial regulation of organ identity genes and/or their regulators. This phenotype also shares characteristics with that of FLIP gene mutations, indicating that the affected gene may have multiple roles throughout flower initiation and development. These hypotheses are tested through double mutant analyses and observation of the effect of ufo mutations on FLIP and organ identity gene transcript patterns. Finally, I describe how, in collaboration with Dr. Enrico Coen, UFO was isolated and how subsequent characterization of its transcript patterns throughout flower development in both wild type and mutant plants indicates possible functions for the gene product.
2. MATERIALS AND METHODS

2.1 Genetic and Phenotypic analyses of Arabidopsis

2.1.1 Plant material

The UNUSUAL FLORAL ORGANS mutant line Ufo-1 was isolated by Dr. George Haughn from an ethylmethane sulfonate-mutagenized population of Arabidopsis thaliana ecotype Columbia-2 (Col-2). Ufo-1 was back-crossed to the wild type a minimum of four times prior to phenotypic analysis of mutants segregating from backcross populations. The line segregating ufo-2 was a gift of E. M. Meyerowitz (California Institute of Technology, Pasadena, CA), and lines carrying the alleles ufo-3 (SI340), ufo-4 (SI341), and ufo-5 (SI747) were gifts of D. Smyth (Monash University, Clayton, Australia). The lines W100 (angustifolia [an], apetala1 [ap1], pyrimidine requiring [py], erecta1 [er1], long hypocotyl [hy2], glabrous1 [gl1], eceriferum2 [cer2], brevipedicellus [bp], male sterile [ms], transparent testa [tt3]; Koornneef, et al., 1987), MSU7 (eceriferum5, [cer5], distorted trichomes2 (dis2), gibberellic acid resistant (ga4), er1; Koornneef and Hanhart, 1983), Agamous-1 (Ag-1), Ap1-1, Apetala2-1 (Ap2-1), Pistillata-1 (Pi-1), and Apetala3-1 (Ap3-1) were gifts from M. Koornneef (Wageningen Agricultural University, The Netherlands). Lines Clavata1-1 (Clv1-1) (NW45), and Clavata2-1 (Clv2-1) (NW46) were obtained from the Arabidopsis Biological Resource Centre (Ohio State University, Columbus, OH). Other mutants used were SAS 1-3-7 (Ap2-6) (Kunst, et al., 1989), Leafy-1 (Lfy-1) (Haughn and Somerville, 1988; Schultz and Haughn, 1991), Leafy-2 (Lfy-2) (Schultz and
2.1.2 Growth conditions

Seeds were germinated at 4°C for 3 days in 5-in diameter pots containing Terra-Lite 'Redi Earth' prepared soil mix (W.R. Grace & Co. Canada Ltd., Ajax, Ontario, Canada) and then transferred to growth chambers at 22°C and continuous light (CL) 90-120 μE m² sec⁻¹ or short day (SD) (10 hr light/14 hr dark, 130 μE m² sec⁻¹). For floral morphology studies, plants were grown at a density of approximately 50-70 per pot. Plants grown for inflorescence structural analysis had a density of 14 per pot. For SD experiments seven plants were grown in each pot.

2.1.3 Microscopy

A minimum of 14 flowers from at least 4 plants were selected for each single or double mutant combination. Fresh tissue was dissected and examined under a dissecting microscope to determine morphological characteristics.

For scanning electron microscopy (SEM), partially dissected tissue was fixed in 3% glutaraldehyde in a 0.1 M phosphate buffer, pH 7. Fixed tissue was then dehydrated in an acetone series and critical point dried. Each sample was stuck to aluminum SEM stubs with double-sided tape and dissected further using pulled glass needles before being gold coated in an Edwards S150B sputter coater. Dissection of Ufo-1 Ap2-6 double mutant flowers involved fracturing the flower to generate cross-sectional views prior to sputter
coating. Observations were made using a Phillips model 505 Scanning Electron Microscope with an accelerating voltage of 30 kV.

For confocal microscopy, fresh tissue was dissected and fixed in 70:10:5 Ethanol/Formaldehyde/Propionic acid overnight at room temperature. Tissue was then immersed in Ethanol to clear, then gradually rehydrated through an ethanol/water series into distilled water. Rehydrated tissue was stained in 0.2 ml of 5mg/ml Propidium Iodide in 0.1M L-Arginine pH 12.5 for several hours. Tissue was then destained extensively (several days) in 0.1M L-Arginine pH 8.0. Tissue was most conveniently viewed by placing in a thick fluid such as glycerol in a well-slide, and covering with a coverslip.

2.1.4 Linkage analysis

Linkage analysis was performed using the LINKAGE-1 computer program (Suiter et al., 1983). The ufo-1 mutation was initially mapped to chromosome 1 by examining the phenotypes of 316 plants from the cross ufo-1/ufo-1 X W100. ufo-1 was then crossed to the chromosome 1 marker line MSU7 (eceriferum5, distorted trichomes2, gibberellic acid resistant4; erectal). Of 535 F2 plants, none showed a recombination event between ufo-1 and the dis2 marker, indicating that these two loci were closely linked (maximum 9.50 map units +/-4.28 map units). One hundred lines from individual Dis2 plants in this population were scored for segregation of the Ufo phenotype. A single line segregated Ufo Dis2 Ga4 triple mutants indicating that the map distance between UFO and DIS2 was approximately 0.5 map units with the UFO locus between CER5 and DIS2.
2.2 Construction and identification of double mutant plants

Double mutants were identified by analysing segregation ratios and test crossing. The details of each cross are presented in the next section. When double mutants could be identified, a minimum of 13 double mutant plants were examined. The exception was Ufo Pi, which is a double mutant, which can only be identified by test crossing. Five double mutants were confirmed in this manner.

Ufo-1 Pi-1:
Homzygous pi-1(Ler) pollinated by ufo-1 homozygotes produce wild type F1 plants. The F2 segregated wild type (WT) and mutant plants in an approximate 9:7 ratio (73WT: 67 mutant, $X^2= .959$, df=1, $p>0.25$). Phenotypic variability made it difficult to absolutely distinguish between Pi-1, ufo-1, and Pi-1 ufo-1 double mutant plants with certainty. Nineteen mutant plants were selected that included all floral phenotypes. These phenotypes were recorded and the flowers pollinated by ufo-1 homozygotes, and pi-1/PI heterozygotes. Of 16 successful test crosses, 5 double mutant, 5 Pi, and 6 Ufo individuals were confirmed.

Ufo-1 Ap3-1:
Ufo-1 plants pollinated by ap3-1 (Ler) homozygotes produced wild type F1 plants. F2 plants segregated mutant phenotypes in an approximate 9:7 ratio (117 wild type:96 mutant $X^2= .15$, df=1, $p>0.5$). Phenotypes of mutant individuals were recorded before test crossing to Ufo-1 and Ap3-1 plants. Of the few successful crosses, phenotypic
characteristics consistently segregating with double mutant plants were identified. To assist in the generation of double mutant individuals, self seed was collected from 8 Ap3-1 plants in an F2 population grown at 16°C, the permissive temperature for the \textit{ap3-1} allele. Two of these lines segregated only Ap3-1 plants when grown at 16°C, however the remaining 6 lines segregated a novel phenotype matching that of the confirmed double mutant phenotype in an approximate 3:1 ratio (120 Ap3-1:27 other, $\chi^2 = 3.45$, p > 0.1).

\textbf{Ufo-1 Ap2-1:}

Ufo-1 flowers pollinated by Ap2-1 produced wild type F1 plants and an F2 population that segregated four distinct phenotypes in an approximate 9:3:3:1 ratio (175 WT:51 Ufo-1:53 Ap2-1:14 other, $\chi^2 = 1.996$, df=3, p > 0.5). The novel class was often male and female sterile, thus only two test crosses were successful. Test crosses of novel individuals to Ufo-1 or Ap2-1 (male parents) generated only mutant offspring confirming that these represented the double mutant class. Of self seed from 6 individual Ap2-1 plants in the F2 population, 5 lines segregated Ap2-1 and the double mutant phenotype in a 3:1 ratio (155 Ap2-1:45 other) and these were used in further phenotypic analyses.

\textbf{Ufo-1 Ap2-6:}

Ufo-1 flowers pollinated by Ap2-6 produced a wild type F1 and an F2 segregating three distinct phenotypes in an approximate 9:3:4 ratio (106 WT:37 Ufo:43 Ap2-6 $\chi^2 = .411$, df=2, p > 0.75), suggesting that the double mutant phenotype was similar or identical to that of Ap2-6, although the alternate hypothesis that the double mutant is Ufo-like (9 wild type: 4 Ufo: 3 Ap2-6) can not be rejected ($\chi^2 = 3.85$, df=2, p > 0.1). Self seed from 16
of 24 ufo individuals segregated an Ap2-6-like phenotype in an approximate 3:1 ratio (73Ufo:33Ap2-6-like, $X^2=2.12$, df=1, $p>0.05$) indicating that these novel Ap2-like plants represented the Ufo-1 Ap2-6 double mutant class. Differences between these flowers and those of Ap2-6 plants were apparent in this population. An F2 population examined by these criteria segregated four phenotypes in ratio of 44WT:17 Ufo-1:19 Ap2-6:10 other ($9:3:3:1$, $X^2=4.53$, df=3, $p>0.1$).

Ufo-1 Ag-1:

Ufo-1 flowers were pollinated by several wild type flowers from a population segregating the Ag-1 phenotype. Individual male parents were allowed to self and only crosses to plants segregating Ag were examined further. F1 plants were wild type, and individual F2 populations segregating Ag plants were examined for novel phenotypes. These F2 lines segregated four distinct phenotypes in an approximate 9:3:3:1 ratio (152WT:59Ufo-1:51Ag-1:19other, $X^2=1.16$, df=3, $p>0.75$) suggesting that the novel phenotype represents the Ag-1 Ufo-1 double mutant class. In support of this hypothesis, self seed from 6 Ufo-1 plants in the F2 generation generated 2 lines that segregated the novel phenotype in a ratio of 16Ufo:8other.

Ufo-1 Apl-1:

The wild type F1 from Ufo-1 flowers pollinated by apl-1 homozygotes produced an F2 that segregated four phenotypic classes in an approximate 9:3:3:1 ratio (208WT:72Ufo-1:78Apl-1:13other, $X^2=5.58$, $p>0.1$). Plants exhibiting the novel phenotype were test crossed to Ufo-1 and Apl-1 parents. Of 4 successful crosses to Ufo-1, all progeny were
phenotypically Ufo-1. Likewise, 3 successful crosses to Ap1-1 resulted in Ap1-1 progeny only. Test crossing Ap1-1 and Ufo-1 plants from the F2 generation indicated that none were homozygous for both mutations. These results support the designation of the novel class as the Ufo-1 Ap1-1 double mutant.

Ufo-1 Flo10:

Ufo-1 plants pollinated by Flo10 flowers produced a wild type F1. The F2 segregated four distinct phenotypes in a ratio of 406WT:119Ufo:100Flo10:38other. The novel phenotypic class was sterile. Four of 6 Flo10 plants test crossed to Ufo-1 segregated the Ufo-1 phenotype in the next generation. These individuals (ufo-1/ufo-1; FLO10/flo10) were allowed to self and the progeny scored. All plants segregated both the Ufo-1 phenotype and the novel phenotypic class in an approximate 3:1 ratio (63Ufo:20other) suggesting that the novel phenotype is that of Ufo-1 Flo10.

Ufo-1 Tfl1-14:

Ufo flowers were pollinated by a tfl1-14 homozygote generating a wild type F1 generation that produced an F2 segregating four distinct phenotypes in an approximate 9:3:3:1 ratio (194wt:69Ufo:60Tfl:17other; $X^2 = 1.542$, $p > 0.5$). Lines were derived from self fertilization of individual Tfl plants in this generation that segregated Tfl and the novel phenotype in an approximate 3:1 ratio (55Tfl:20other) indicating that the novel phenotype represents that of the double mutant class.

Ufo-1 Lfy-2:
Stamens from a Lfy-2 plant were used to pollinate a Ufo-1 flower. The F1 generation was entirely wild type, and the F2 generation segregated three distinct phenotypes in an approximate 9:3:4 ratio (352wt:111Ufo:145leafy-like; $X^2 = 6.93$, $p > .50$, d.f. = 2; alternate hypothesis 9:4:3 gives $X^2 = 19.78$, $p < .01$). Among the leafy-like progeny were individuals resembling the Lfy-2 parent and individuals exhibiting stronger leafy-like transformations, which may represent the double mutant phenotype. Seed from Ufo-1 plants in this population were tested and all such lines were found to be the result of outcrosses. Due to the phenotypic variability of Lfy-2 plants, and the sterility of the putative double mutant class we were unable to identify double mutant individuals. A second cross between lfy-2 and the linked marker line ufo-1 dis2-l resulted in an F2 population that segregated Leafy plants with distorted trichomes (dis2 homozygotes). These individuals were presumed to represent Ufo-1 dis2-l lfy-2 triple mutant individuals, and the Ufo-1 lfy-2 double mutant phenotype was inferred from analysis of these individuals.

**Ufo-1 Lfy-1:**

Several wild type plants from a line segregating Lfy-1 were used to pollinate Ufo-1. Crosses from those wild type individuals that segregated the Lfy-1 phenotype in the next generation were retained. Seed from these crosses generated a wild type F1 and individual plants were harvested. Lines segregating Leafy-like plants in the F2 were examined further. Three phenotypes were observed in an approximate 9:3:4 ratio (72WT:16Ufo-1:21Lfy-1, $X^2 = 4.25$, $p > 0.1$) however the alternate hypothesis of 9:4:3 can not be rejected by these data.
2.3 *In situ* hybridization of *Arabidopsis* tissue

2.3.1 Preparation of plant material

*In situ* hybridization was performed using an adaptation of Huijser, et al. (1992). Freshly dissected whole apices were vacuum infiltrated with FAA (3.7% paraformaldehyde, 5% acetic acid, 50% ethanol in water) for several hours. Infiltrated tissue was left to fix overnight at 4°C. Fixed tissue was dehydrated through 70%, 90% and 100% ethanol, then cleared through 25%, 50%, 75%, and 100% xylene in ethanol. Chips of Paraplast Plus (Sigma) were added to xylene and left to dissolve overnight. Samples were placed at 60°C and the xylene was replaced stepwise by molten paraffin over two days. Samples were left at 60°C until embedding. Embedding was done in 2" plastic petri dishes, and embedded samples were left to harden on ice for several hours before storage at room temperature.

Embedded samples were mounted on wooden blocks and 7μm sections were floated on 45°C water before being placed on slides pre-treated with Vectabond adhesive (Vector Labs). Sections were fixed to the slides by heating to 65°C for one hour.

2.3.2 Preparation of Probes

The following plasmids were used: pCIT565(*AG*; Yanofsky, et al., 1990), pD793(*AP3*; Jack, et al., 1992), pKY89(*AP1*; Gustafson-Brown, et al., 1994), pcAT10-105(*AP2*; Jofuku et al., 1994), PJAT170(*UFO*; Ingram et al., 1995), and pcPINX(*PI*; Goto
and Meyerowitz, 1994). The plasmid pDW124 containing a portion of the \textit{LFY} coding sequence was provided by D. Weigel (Salk Institute for Biological Studies, La Jolla, CA). Each plasmid was cleaved at the 5' end of the insert (pCIT565-HindIII, pD793-BglII, pKY89-XhoI, pcPINX-NsiI, pclT10-105-EcoRI, and pDW124-BamHI) and transcribed in the presence of Digoxigenin-labelled nucleotides to generate an antisense transcript which was hydrolysed to generate fragments of approximately 150 nucleotides.

Digoxigenin labelling of probes, antibody detection, and staining with 4-nitro blue tetrazolium chloride (NBT)/X-phosphate was performed using Boehringer Mannheim methods and reagents (kit #1175-041).

2.3.3 In situ hybridization and detection

Slides were racked in glass staining dishes and passed through the following series: 100% xylene twice for ten minutes, 1:1 xylene/ethanol for 10 minutes, and 100% ethanol twice for 5 minutes each to remove paraffin. Sections were then re-hydrated over a period of 20 minutes by passing through a series of 75% ethanol, 50% ethanol, 25% ethanol, followed by two .85% NaCl washes. Slides were immersed in pre-heated 2XSSPE (1X = 0.15M NaCl, 0.01M NaPO$_4$, 0.001M EDTA, pH 7.4) at 70°C for 1/2 hour. Proteinase digestion was performed on the sections by immersing the rack in 37°C Proteinase K (1 \mu g/ml Proteinase K in 10 mM Tris-HCl, 50 mM EDTA pH8 buffer) for 1/2 hour, followed by three washes in Phosphate-buffered saline (PBS) (8g NaCl, 0.2g Kcl, 1.44g Na$_2$HPO$_4$, 0.24g KH$_2$PO$_4$ in 1000ml water pH 7.4). Slides were then dehydrated through an ethanol series to 100% ethanol and allowed to dry thoroughly.
Antisense probe was incubated at 90°C for 5 minutes and added to a final concentration of .05 ng/µL in hybridization buffer (Hybridization Buffer: 100 µL 10X in situ salts, 50% Deionized formamide, 1000 µg/ml tRNA, 100 µg/ml poly(A) RNA, 1X Denhardts, 10% Dextran Sulfate) (10X In Situ Salts: 3 M NaCl, 0.1 M Tris-HCl, 0.1 M NaPO₄ buffer pH 6.8, 5 mM EDTA). 100µL of hybridization solution was added to each slide and spread under a glass coverslip. Slides were laid flat and allowed to hybridize at 50°C overnight. Coverslips were then removed and slides were immersed in pre-heated 50°C 1:3 Formamide/4XSSPE for one hour, followed by several washes in PBS. Slides were blocked using 1X Boheringer Mannheim blocking reagent (1% Boehringer blocking agent in 100mM Maleic Acid, 150mM NaCl pH7.5) for one hour, followed by a one hour wash in BSA-Triton buffer (1% Bovine Serum Albumen (BSA), 0.3% TritonX-100, 100mM Tris-HCl pH7.5, 150mM NaCl). Antibody was diluted 1:1500 in BSA-Triton buffer and spread evenly over the sections. Slides were incubated for 4 hours, followed by two hour-long washes with clean BSA-Triton buffer to remove unbound antibody. Color reagents were made up following manufacturers instructions, and spread over slides. Color was allowed to develop in the dark for 24 hours, followed by several washes in distilled water.

After detection, slides were dehydrated through ethanol and xylene and mounted in Entellan mounting media (Merck). Sections were photographed through a Leitz DRB (Leica, Wetzlar, Germany) light microscope using Kodak T160 ASA film.
3. CHARACTERIZATION OF THE UFO PHENOTYPE

Here I present an analysis of the Unusual floral organs (Ufo) mutant phenotype. Together, the data collected suggest that the affected gene appears to have a role in both floral initiation and the regulation of gene expression boundaries within the flower. Much of this work has been previously published by the author (Ingram et al., 1995; Wilkinson and Haughn, 1995; Wilkinson and Haughn, 1994; Haughn et al., 1994). In addition, a similar independent investigation of the Ufo phenotype was recently published (Levin and Meyerowitz, 1995). Some discrepancies exist between our results and those of Levin and Meyerowitz, and these will be addressed where appropriate throughout the remainder of this work.

3.1 Review of wild type floral and inflorescence features affected by ufo

*Arabidopsis* flowers consist of a basal rosette and an aerial shoot from which coflorescences and flowers arise. Two features of wild type are significant to the discussion of the Ufo phenotype.

First, the change in lateral shoot fate from coflorescence to flower is abrupt and permanent in wild type plants. This appears to result from coordinated activation of the FLIP genes at a certain threshold of COPS activity, and subsequent inhibition of COPS activity in the (now determined) floral meristem. Although this decision appears to be propagated apically, in that all future lateral shoots also develop as flowers, it is not clear how this occurs. It is possible that, once active, FLIP genes in one node signal the
subsequent node to be floral. Alternately, it is possible that each node 'interprets' the COPS signal independently and, below a certain threshold of COPS activity, FLIP genes are always activated, resulting in a precise transition from coflorescence to flower production.

Second, flowers from wild type plants are all identical in organ number, type and position. This is associated with a precise patterning of floral homeotic gene transcripts and products within the developing floral meristem. It is known from mutant phenotypes, and from transgenic studies, that disruptions in wild type organ identity and pattern occur when organ identity genes are not expressed or expressed ectopically. Thus the regulation of organ identity gene boundaries is, clearly, critical for normal flower development. There are at least three boundaries that must be accounted for. The boundary between class A and C genes (this is considered a single boundary since they appear to be mutually exclusive and antagonistic), the first/second whorl boundary of class B gene activity, and the third/fourth whorl boundary of class B gene activity. The latter of these is dependant in part on the expression of the FLO10 gene product, which is expressed on the third/fourth whorl boundary and has been proposed to act as a regulator of cell division, preventing class B-expressing cells from entering the fourth whorl (Sakai et al., 1995). However, this explanation then makes it necessary to account for the spatial regulation of FLO10 itself. It is possible that all of these boundaries are controlled through a single mechanism, or that they are controlled individually.
3.2 Isolation and mapping of Ufo alleles

The Ufo phenotype appeared in a population of EMS-mutagenized plants. The phenotype included dramatic and unusually variable changes in organ type in the second and third whorls. Single plants exhibiting this phenotype were backcrossed to the wild type over 5 generations and, after each generation, the phenotype was observed. Several phenotypic features were apparent in some Ufo plants from early backcross populations but were not present in later backcrosses, indicating that they were the result of mutations at other loci, however the overall phenotype remained quite variable. Wild type and Ufo plants segregated in an approximate 3:1 ratio (719 wild type: 243 Ufo; $X^2 = 0.55$, df = 1, $p > 0.9$) in the F2 of the fourth backcross, indicating that Ufo results from a single nuclear recessive mutation in the gene designated \textit{UNUSUAL FLORAL ORGANS} (\textit{UFO}). The mutant allele is designated \textit{ufo-1}.

The variable nature of the Ufo-1 phenotype remained even after 6 backcross generations. Presuming that one half of the genome is replaced by wild type material in each backcross, only 1.6\% of the mutagenized genome should remain in the F2 from the sixth backcross, thus it is unlikely that this variability results from segregation of mutations at other loci, but rather is a feature of the Ufo-1 mutant phenotype itself. This is supported by the availability of four additional alleles, designated \textit{ufo-2, ufo-3, ufo-4}, and \textit{ufo-5} (see materials and methods for the history of these alleles), all of which generate highly variable floral organ transformations. Given the number of recessive alleles that have identical phenotypes, it is likely that this phenotype results from a loss of \textit{UFO} gene function. This is supported by sequence data (see section 5.4).
Figure 3.1. Map Position of the *UFO* Gene on Chromosome 1. Results of linkage analysis from the cross *ufo-1/ufo-1* X *MSU7* (*Ler; ga4 dis2 cer5; erl*) are shown. Numbers below the arrows represent distances between markers in map units.
Mapping of the *ufo-1* locus was achieved through a series of crosses to multiply-marked mapping lines. Ufo-1 plants were crossed to the mapping line W100, which carries one mutation on each arm of each of the 5 *Arabidopsis* chromosomes. Linkage analysis placed the *UFO* locus on chromosome 1, linked to *ANGUSTIFOLIA*. *UFO* was more precisely mapped using the chromosome 1 marker line MSU7 (*eceriferum*5 (*cer5*), *distorted trichomes*2 (*dis2*), *gibberellic acid resistant*4 (*ga4*); *erecta* (*er*)) (Koornneef and Hanhart, 1983). Ufo displayed linkage to three markers on this chromosome: *CER5*, *DIS2*, and *GA4*. Mapping data is diagrammed in Figure 3.1. No recombinants were detected between *ufo* and *dis2* in the F₂ of this cross. 100 Dis2 plants from this F₂ population were allowed to self fertilize, and examined for segregation of the Ufo phenotype. One line segregated both Dis2 and Ufo Dis2 double mutant plants in a 3:1 ratio, indicating that one of the 100 plants was heterozygous for a chromosome recombinant between *UFO* and *DIS2*. Thus the map distance between *UFO* and *DIS2* is approximately 0.5 map units (1 recombinant chromosome out of 200 chromosomes). No other genes affecting floral or inflorescence morphology have been mapped to this location.

### 3.3 Ufo floral morphology

The *ufo-1* allele was generated in the Columbia-2 (Col-2) ecotype, while all other available alleles are from the Landsberg *erecta* (*Ler*) ecotype. Most Ufo phenotypic
Table 3.1 Numbers of Different Organ Types in Second and Third Whorls of Ufo-1 Flowers

<table>
<thead>
<tr>
<th>Whorl</th>
<th>Petal Filament-like organ</th>
<th>Petal-sepal</th>
<th>Petal-stamen</th>
<th>Stamen</th>
<th>Carpels</th>
<th>Sepals</th>
<th>Absentb</th>
<th>Additionalc</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd</td>
<td>109</td>
<td>102</td>
<td>92</td>
<td>55</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>28</td>
</tr>
<tr>
<td>3rd</td>
<td>5</td>
<td>424</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>13</td>
<td>0</td>
<td>164</td>
</tr>
</tbody>
</table>

*a*organs in unambiguous positions were scored for organ type and whorl position in 106 flowers grown at 22°C. Organs in ambiguous positions were not scored.

*b*Absent indicates the total number of missing organs in the indicated whorl for all flowers scored.

*c*Additional indicates the frequency of additional organs appearing within a whorl.

features are present in alleles from both ecotypes, thus the Ufo-1 phenotype can be considered representative. In cases where this is not true, reference will be made to any differences between the alleles.

Table 3.1 and Figure 3.2 illustrate the highly variable floral morphology of Ufo-1 plants. Homeotic changes are apparent in second and third whorl organs, whereas changes in organ number occur in all whorls. Floral defects are most severe in early flowers (Figure 3.2D) but become less pronounced apically. The last flowers produced may exhibit a completely wild type morphology. Unlike most other mutations affecting floral organ type, the fate chosen by an organ primordium can differ from that chosen by other primordia in the same whorl. Because of this variability it is not feasible to describe a 'typical' flower, rather it is likely that the variability is a significant phenotypic feature that provides information about the role of the UFO gene in wild type flower development.

Ufo-1 flowers generally produce four first whorl organs consisting primarily of sepal tissue. ufo alleles in the Ler ecotype occasionally generate first whorl organs that are
mosaics of sepal and carpel cell types (15 organs of 76 flowers scored) and may fuse to each other or to organs from other whorls. This becomes more pronounced in the most apical flowers. Alleles in all ecotypes generate additional first whorl sepals at low frequency (two in 106 flowers); however, ontogenic analysis indicates that pairs of sepal primordia frequently appear in the position expected of a single primordium in the wild type. To account for the discrepancy between the ontogenic analysis and the mature phenotype, it seems likely that these pairs of primordia fuse shortly after initiation resulting in a single sepal.

Second whorl organ types in Ufo-1 flowers range from sepals and filamentous organs (similar to Figure 3.2J) in early flowers to normal petals or staminoid-petals in later flowers. Typical second whorl organs are shown in Figures 3.2G and 3.2H. Relative numbers of obvious mosaic variations are listed in Table 3.1. On the basis of cell surface features, most second whorl organs are mosaics of two or more organ types; every floral organ type has been observed in these mosaic structures, although carpel tissue is only rarely observed. Early flowers have fewer second whorl organs, and ontogenic analysis indicates that individual second whorl organ primordia are frequently absent when compared to the wild type. The absence of a second whorl organ primordium does not alter the placement of other second whorl primordia. No morphologically normal stamens ever appeared in the second whorl, although locules on petal-stamen mosaic organs could dehisce to produce pollen.
Figure 3.2: Morphology and development of Ufo-1 plants.

(A) Inflorescence of Ufo-1 plant grown in SD. Arrows indicate two coflorescence nodes that are separated by two flowers (one flower is hidden from view).

(B) Apical nodes of Ufo-1 plants grown in SD conditions. Arrows point to bracts subtending Ufo-1 flowers.

(C) Floral node from Ufo-1 plants grown in SD from which no lateral shoot was produced. Stipules [s] and bract-like structures [b] are visible.

(D) Typical mature Ufo-1 flower.

(E) Inflorescence of Ufo-1 plant grown in CL. Additional sepal primordia are visible on some developing flowers (arrows).

(F) Developing Ufo-1 flower. An organ primordium is missing from the third whorl position (arrow). Other primordia are numbered according their position in the second [2] or third [3] whorl.

(G) Second whorl organ from Ufo-1. Regions of petal [P], and sepal [S] tissue are visible.

(H) Second whorl organ from Ufo-1. Regions of petal [P], sepal [S], and stamen [T] tissue are visible.

(I) Third whorl organ from Ufo-1. Stigmatic papillae [p] typical of carpels are visible at the tip of this organ.

(J) Filamentous organ from the third whorl of a Ufo-1 flower.

(K) Developing Ufo-1 gynoecium. Septa primordia have fused to generate three ovarian chambers.

(L) 'Empty flower' from Ufo-1 plant.

Typical third whorl organs are represented in Figures 3.2I and 3.2J. The relative numbers and types of transformations are listed in Table 3.1. The most frequent organs are filamentous, however they are taller and thicker than filamentous organs in the second whorl and are occasionally capped with stigmatic papillae (one of 106 flowers scored). Rare organ types include staminoid-carpels and, in later flowers, completely normal petals and stamens. Variations in organ number, both greater and fewer than six, were common in the third whorl. Organs could be missing from any third whorl position, however when additional organs were produced they invariably appeared between a pair of medial primordia. The addition or loss of organ primordia did not affect the position of other primordia within that whorl (Figure 3.2F).

Second and third whorl filamentous organs have no clear wild-type counterpart. The length of the organ is composed of cells with surface features typical of a stamen filament while, at the tip, cuticular thickenings similar to carpel, anther or nectary cell types are present (Figure 3.2J). Cross-sections revealed that, unlike stamen filaments, these organs are never vascularized.

Frequently, when a third whorl organ is absent, the adjacent second whorl organ exhibits a mosaic sector containing stamen tissue along its closest margin. Although this correlation is not absolute, it is possible that at least some organ mosaics represent fusions between organ primordia in different whorls. However, since primordia fusion was never observed ontogenically, this event would have to occur very early in primordium initiation.

The fourth whorl consists of two to four fused carpels (average of 2.98 with a modal number of 3 from 106 flowers scored) and/or filamentous organs. In many cases
ovaries from one or more carpels do not extend the full length of the gynoecium. The presence of extra carpels does not consistently correlate with organ loss from any other whorl, suggesting that it is additional fourth whorl primordia, rather than fusion of outer whorl primordia with the fourth whorl, that accounts for the majority of these additional carpels. In support of this, ontogenic analysis indicated that up to four fourth whorl primordia are initiated (Figure 3.2K).

In addition to the 'structurally normal' Ufo-1 flowers just described, lateral shoots consisting solely of two to three sepals appear with a frequency of approximately one to two per plant (Figure 3.2L). Flowers produced immediately before and after these unusual 'empty flowers' show no obvious differences from the typical Ufo-1 floral phenotype. SEM analysis revealed that no additional organ primordia are initiated within these flowers.

Ufo-1 flowerlike shoots that developed under SD conditions differ only slightly from those of plants in CL. Petal-stamen mosaic organs are rare, although sepal-stamen mosaic organs and sepal-carpel mosaic organs appear occasionally in second and third whorl positions. Fourth whorl carpels frequently were not fused. Other alleles of ufo, however, produce additional whorls of floral organs in SD. Up to four additional sepals were observed in Ufo-2, apparently forming a whorl interior and alternate to the second whorl sepaloid organs. A further whorl of stamens, staminoid-carpels, and filamentous organs (similar to the third whorl of Ufo-1) was generated prior to the production of a gynoecium. This phenotype resembles that of the fimbriata mutation from Antirrhinum majus, which has been shown to be homologous to the UFO gene (see chapter 6).

Changes in organ number have been associated with changes in meristem size (such
as in Clavata mutant flowers; Leyser and Furner, 1992; Clark, et al., 1993). Measurement of Ufo floral and apical meristems revealed that the mean diameter of Ufo-1 floral meristems measured by both SEM and confocal scanning microscopy was consistently higher than that of wild type, however the differences were not always statistically significant. Similarly, the mean diameter of inflorescence meristems was consistently higher in Ufo plants, but the differences were not statistically significant.

3.4 Ufo inflorescence morphology

3.4.1 Ufo inflorescences in continuous light

Figures 3.2 and 3.3 and Table 3.2 provide data concerning the rosette and inflorescence morphology of the Col-0 and Ler wild type ecotypes of Arabidopsis, and two Ufo mutant phenotypes, Ufo-1 (Col-0), and Ufo-3 (Ler). When under CL conditions, Ufo

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Phenotype</th>
<th>Number of rosette leaves</th>
<th>Number of coflorescences</th>
<th>Number of plants scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>Ufo-1</td>
<td>9.1 ± 0.96</td>
<td>3.5 ± 0.63</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Col-2</td>
<td>8.8 ± 0.51</td>
<td>2.5 ± 0.60</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Ufo-3</td>
<td>7.0 ± 0.53</td>
<td>2.8 ± 0.81</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>7.4 ± 0.83</td>
<td>1.6 ± 0.51</td>
<td>15</td>
</tr>
<tr>
<td>SD</td>
<td>Ufo-1</td>
<td>62.4 ± 8.95</td>
<td>15.9 ± 2.92</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Col-2</td>
<td>62.3 ± 9.64</td>
<td>10.1 ± 1.80</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Ufo-3</td>
<td>37.8 ± 4.54</td>
<td>14.9 ± 1.27</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>33.1 ± 3.73</td>
<td>8.5 ± 1.22</td>
<td>26</td>
</tr>
</tbody>
</table>

*Figures are given as mean ± standard deviation.

*bSignificant difference from appropriate wild-type strain at P=0.05 level of significance by the students t test.

ccontinuous light

dshort day
mutants exhibit a slight but significant increase in coflorescence number compared to the wild type whereas rosette development is not significantly affected (Figure 3.3A,B; Table 3.2). Unlike the wild type, the meristems of all Ufo primary and coflorescence shoots terminate in a structure resembling either a normal pistil or fused sepal-carpel organs (Figure 3.2M). The number of nodes bearing flowers produced prior to termination in Ufo-1 is approximately the same as the number of floral nodes generated before senescence in wild-type plants; however, alleles of ufo in the Ler background cause some mutants to terminate apical growth as early as the third floral node (Ufo-3: range 3-13 floral nodes, mean 7.9 compared to Ler: range 24-36 floral nodes, mean = 30.3). Thus, although Ufo plants are largely sterile, the termination of Ufo plants is not necessarily analogous to the termination observed in male sterile plants discussed earlier. Such observations suggest that premature termination of apical growth may be a significant aspect of the Ufo mutant phenotype, rather than a pleiotropic effect of sterility.

3.4.2 Ufo inflorescences in short days

Although the effects of Ufo on inflorescence structure were modest under CL, Figures 3.2, 3.3 and Table 3.2 show that vegetative growth of Ufo mutants in SD was strikingly different from that of the wild-type (Figs. 3.2A,B and 3.3C,D). To ensure that these vegetative changes were not allele-specific, two alleles, one in each of two genetic backgrounds, were examined. Mutants generate two to three times more coflorescence nodes than do wild-type plants that bolt during the same interval. Further, Ufo-3 plants generated 26.7% more vegetative nodes than Ler, while Ufo-1 plants generated 8.1% more
vegetative nodes than Col-2. In addition, approximately one to three nodes generated prior to the first flower consist of a bract with no associated axial shoot (Figure 3.3D). Ufo plants in both ecotypes produce coflorescences apical to early floral nodes in both the primary and lateral inflorescence shoots (Figures 3.2A, 3.3D). Up to seven coflorescences can be produced consecutively in positions apical to the first flower before the ensuing flower is produced. These phases of coflorescence production can occur up to three times during the growth of an individual plant, with each coflorescence phase being separated by one to five Ufo-1 flowers. As in CL, all apical meristems terminate in carpel-like structures. Bracts or filamentous structures, and stipules subtended most Ufo-1 flowers. Typically, morphologically normal bracts are limited to the most basal and most apical flowers (Figure 3.2B). In addition, SD-grown plants invariably generate numerous nodes in floral position from which no shoot of any kind was produced (Figure 3.2C). Ten or more such nodes appear consecutively after the first four to five flowers have been initiated and can be identified by the presence of reduced bract-like structures and stipules. Production of Ufo-1 flowers resumes thereafter, however these unusual nodes continue to appear in scattered positions throughout the inflorescence.

3.5 Discussion of Ufo floral and inflorescence features

In general, Ufo flowers exhibit occasional carpelloidy of the first whorl, highly variable transformations in the second and third whorls, and normal organ type in the fourth whorl. These organ type changes could be the result of affects on organ identity
Figure 3.3: Morphology of Ufo inflorescences in CL and SD conditions. Nodes with no lateral shoot are indicated as a bract with no associated inflorescence. Indeterminate inflorescences are indicated by arrows, and determinate inflorescences are indicated by lines without arrows.

(A) Morphology of a wild type plant grown in CL.
(B) Morphology of Ufo-1 plants grown under in CL.
(C) Morphology of wild type plants grown in SD.
(D) Morphology of Ufo-1 plants grown in SD. Note nodes from which no lateral shoot is produced.
gene expression levels or patterns as a result of the *ufo* mutation.

Among the most common organ type transformations in Ufo are petal to sepal and stamen to filament. Class B mutants, such as Pistillata, also exhibit sepaloidy of the second whorl and filamentous structures in the third whorl. Thus it is possible that many of the transformations in Ufo could result from a reduction in the class B gene expression level or domain. However, this is not sufficient to account for all organ type transformations observed in Ufo-1. The appearance of reproductive tissue (both stamen and carpel) in the perianth whorls, and perianth tissue in the reproductive whorls suggests that the boundary between class A and C organ identity gene expression is not consistently established or maintained between whorls two and three. Thus, in Ufo flowers, class C gene expression may appear in some first and second whorl organs, causing transformation to reproductive structures, and be absent from some third whorl organs, resulting in transformation to perianth structures.

The overall variability of the Ufo phenotype, particularly the range of floral homeotic changes, is reminiscent of the phenotypes of weak FLIP mutations such as Lfy-2, and Ap1-12. Increased coflorescence number, increased severity under short-day conditions, and apical termination are all features shared with various FLIP mutants, while the appearance of coflorescences apical to flowers further indicates that floral initiation is affected by the *ufo* mutation. FLIP mutations have a dramatic effect on the expression of class B genes; similarly, many of the Ufo phenotypic features are consistent with a reduction in class B function. Thus, although the effects of *ufo* are primarily floral, it is possible that *UFO* represents a novel FLIP gene.
4. DOUBLE MUTANT ANALYSES

4.1 Hypotheses and predicted phenotypes

At least three hypotheses arise from the Ufo phenotype: 1) \textit{ufo} mutations result in reduced or altered class B gene expression. 2) \textit{ufo} mutations result in changes in the expression levels and/or domains of class A and C genes. 3) \textit{UFO} is involved in floral initiation, and possibly acts in the FLIP mechanism. The degree of variability and organ mosaicism in Ufo flowers, the prevalence of ambiguous organ types such as filaments, and the weak effect of \textit{ufo} on the FLIP in CL make it difficult to observe and measure the effects \textit{ufo} on any of these processes. However, these hypotheses allow us to predict the phenotypes of plants that are doubly mutant for \textit{ufo} and other FLIP and organ identity genes.

These predictions have been tested through construction of a large number of doubly mutant lines. In the following sections, each construct is discussed in the context of which hypothesis is being addressed by that double mutant. Single and double mutant phenotypes are described in detail, followed by an interpretation of the salient features for each double mutant phenotype as they relate to the hypothesis in question.

Because several double mutant constructs required crossing into the \textit{Ler} ecotype, \textit{F}_2 plants of a cross between \textit{ufo-1} (Col-2) and the \textit{Ler} wild type were examined to determine whether the \textit{Ler} genetic background modifies the Ufo-1 phenotype. No significant modifications were detected in the \textit{F}_2 generation of this cross.
4.2 Ufo affects class B gene expression

Ufo floral homeotic changes indicate that ufo mutations reduce the expression level or domain of class B organ identity function. To characterize this further, double mutant lines were constructed between ufo-1 and mutant alleles of the class C gene AGAMOUS (ag-1) and the class A gene APETALA2 (ap2-1 and ap2-6).

4.2.1 Analysis of Ufo Ag double mutants

Mutations in AGAMOUS have been well characterized. In general, Ag-1 flowers exhibit third whorl transformations of stamen to petal and, in place of the fourth whorl, three whorls of organs - sepal, petal, petal - are generated. This pattern repeats several times to generate a nested flower phenotype (Figure 4.1A) (Yanofsky, et al., 1990; Bowman, et al., 1991). These results indicate that AG is responsible for three functions within the flower: 1) control of organ type in the third and fourth whorl, and 2) exclusion of class A activity from the inner whorls, and 3) control of determinacy within the flower.

Double mutant phenotypes between class B (Pi and Ap3) and class C (Ag) genes have been well documented as flowers consisting entirely of sepal-like organs as the result of class A activity throughout the flower. If ufo mutations reduce class B gene expression, the phenotype of Ufo Ag double mutants should be similar to that between pi/ap3 and ag - a flower comprised only of sepals. Removing class C gene expression through the ag mutation should allow observation of ufo effects on class B gene expression in isolation from its effects on the class A/C boundary; however if ufo also affects the overall level of
Figure 4.1: Single and double mutant phenotypes of *ufo-1* and *ag-1*, *flo10-1*, or *apl-1*.  
(A) Mature Ag-1 flower.  
(B) Mature Ufo-1 Ag-1 flower.  
(C) Mature Ufo-1 Ag-1 flower. Mature organs have been removed to reveal the 'fan shaped' floral structure.  
(D) Mature Flo10-1 flower.  
(E) Ufo-1 Flo10-1 flower. First whorl sepal has been removed. Third whorl stamen-carpel [sc] organs are visible.  
(F) Mature Ap1-1 flower.  
(G) Mature Ufo-1 Ap1-1 lateral shoot from a position normally occupied by a flower in wild-type plants. Bracts [b] subtending tertiary shoots [sh] are visible. A pistil like structure can be seen at the apex of the shoot.  
(H) Shoot from apical node of a Ufo-1 Ap1-1 plant. Internodes (arrows) show less elongation than in earlier shoots (compare with Figure 5G) giving the shoot a more flowerlike appearance.  
class A activity, then some primordia will not be exposed to any organ identity gene signal, and will develop as leaves. Thus two possibilities can be predicted for the Ufo Ag double mutant phenotype: (1) flowers consisting only of sepals, or (2) flowers consisting of sepals and leaves.

Ufo-1 Ag-1 flowers have an additive phenotype similar to that of Pi-1 Ag-1 (Bowman, et al., 1989, 1991). Double mutant flowers consist entirely of sepal, sepal-petal, and filamentous organs (Fig. 4.1B). Organ primordia are initiated in wild-type position for up to three whorls before becoming disrupted by enlargement of the floral meristem along one axis. Mature flowers are 'fan shaped' and generate large numbers of organ primordia with no apparent whorled pattern (Fig. 4.1C). In later stages the meristem appears to fasciate into several individual meristems that continue to generate sepal organs. A similar enlargement was observed in Pi-1 Ag-1 floral meristems (J. Bowman, personal communication). Unlike Ufo-1 single mutant plants and all other double mutant combinations examined, apical growth in Ufo-1 Ag-1 plants does not terminate with the production of a pistil-like structure. Rather, a flowerlike structure consisting of numerous whorls of sepal and sepal-carpel mosaic organs is produced at the apex of all inflorescences. Unlike the Ag-1 phenotype where internode elongation occurs between nested flowers (Yanofsky, et al., 1990; Schultz, et al., 1991), no internode elongation is apparent within Ufo-1 Ag-1 flowers, even in the absence of the erecta mutation.

This double mutant phenotype suggests that the UFO gene is an enhancer of class B gene expression, however the appearance of petal organs in the double mutant indicates that class B genes are not entirely dependant on UFO activity. In addition, the unusual
fasciation of the floral meristem indicates that *UFO* directly or indirectly plays a role in maintaining floral meristem integrity.

4.2.2 Analysis of Ufo Ap2 double mutants

Mutations in the *AP2* gene affect all whorls but have strong effects on first and second whorl organ type (Bowman, et al., 1989; Kunst, et al., 1989). The *ap2-6* allele reduces organ number and causes perianth organs to develop as carpel or leaf-carpel mosaics (Figures 4.2G and 4.2H) (Kunst, et al., 1989). The *ap2-1* allele causes first whorl organs to develop as leaves and second whorl organs to develop as petal-stamen mosaics (Figure 4.2M) (Bowman, et al., 1989). Fewer third whorl organs are produced in Ap2-6, and these develop as stamens or carpelloid stamens.

If, as predicted, Ufo flowers have reduced class B gene expression, double mutants between *ufo* and the class A gene *ap2* should have a phenotype similar to that of *pi/ap3* and *ap2* double mutants where all organs develop as carpels. Alternately, if *ufo* also causes reduced class C activity, some primordia will develop as leaves.

Flowers of Ufo-1 Ap2-6 and Ufo-1 Ap2-1 double mutants exhibit an additive phenotypic interaction similar to that described for Pi-1 Ap2-2 and Pi-1 Ap2-1 double mutants (Bowman, et al., 1989, 1991). Outer whorl organs of Ufo-1 Ap2-6 flowers are similar to those of Ap2-6 flowers but invariably fuse to enclose the remaining floral organs (Figure 4.2I). In the earliest stages, flowers are composed of two fused carpels (Figure 4.2K). As these flowers mature, additional carpels appear. The additional organs most likely arose from the third and fourth whorls, although I was unable to determine their
Figure 4.2: Single and double mutant phenotypes of ufo-1 and pi-1, ap3-1, ap2-6, or ap2-1.

(A) Pi-1 Flower. Second whorl sepals [s] and third whorl carpels [c] are visible.
(B and C) Ufo-1 Pi-1 double mutant flowers. In (B), sepal/carpels [sc] are fused in a gynoecial-like structure. In (C), a slender, solid gynoecial structure [g] is visible with ovary tissue in the upper regions. Some perianth tissue has been removed for clarity.
(D) Flower from an Ap3-1 plant grown at 16°C. Second whorl sepals [s] and third whorl stamen-carpel organs [sc] are visible. The front sepal has been removed for clarity.
(E and F) Flowers from Ufo-1 Ap3-1 double mutant plants grown at 16°C. In (E), carpel-like organs (3), apparently arising from the third whorl, are seen fused in the center of the flower. The front sepal has been removed for clarity. In (F), Two sepal-like organs (arrows) are visible in typical third whorl positions.
(G) Apetala2-6 flower. Carpels [c] develop in first and second whorl positions.
(H) Apetala2-6 flower showing fusion of all perianth whorl organs.
(I) Ufo-1 Ap2-6 flower. Trichomes (arrows) typical of leaf tissue are visible on the fused outer carpelloid organs.
(J) Developing Ufo-1 Ap2-6 flower. Additional carpels (arrow) appear late in flower development.
(K) Developing Ufo-1 Ap2-6 flower in cross-section. Two ovary chambers are visible at this stage of development.
(L) Developing Ufo-1 Ap2-6 flower in cross-section. This flower is older than the one shown in (K). Four outer ovary chambers are visible surrounding two inner ovary chambers.
exact origin (Figure 4.2J). Mature flowers typically consist of four fused outer and two fused inner carpels (Figure 4.2L).

Ufo-1 Ap2-1 flowers (Figure 4.2N) generate four first whorl primordia and two to four large second whorl primordia that develop as leaf or leaf-carpel mosaic organs. Third and fourth whorls generate small numbers of primordia that fuse soon after initiation, making individual whorl designation difficult. These primordia develop to produce a fused or open multicarpellate structure in the position of the gynoecium.

Since Ufo Ap2 plants resemble Pi Ap2 and Ap3 Ap2 plants, the data support the hypothesis that UFO is an enhancer of class B activity.

4.3 Ufo exhibits changes in the class A/C boundary

4.3.1 Apparent effects of ufo on the class A/C activity

One of the most interesting aspects of the Ufo floral phenotype is the effect of ufo on the class A/C boundary. This effect is likely obscured in Ufo flowers by the presence of 'filamentous' organs that have no wild type counterpart, and thus do not imply any particular combination of class A/B/C activities. It is clear from the floral phenotype that ufo does not result in a complete loss of class B function, thus organ type changes due to class A/C boundary fluctuations may be more easily observed in the absence of all class B activity; double mutants between ufo and the class B genes pi or ap3 should produce only two organ types - sepals and carpels. If, as expected, ufo affects the boundary between class A and C function, then sepal tissue should appear in the reproductive whorls and
carpel tissue should appear in the perianth whorls of double mutant flowers. In addition, 
*ufo* mutations should enhance the phenotype of weak class B mutations such as Ap3-1.

Additional observations may be made in Ufo-class B double mutants. Petal-stamen 
mosaics are a common organ type in Ufo flowers, and previous work (see discussion of 
wild type flower development) has shown that the phenotype class B activity alone may 
specify formation of this organ type. Class A and C expression are usually observed in 
complementary, exclusive domains, and thus share a common boundary; however in Ufo it 
is possible that these petal-stamen organs represent regions where neither class A nor class 
C genes are expressed. If this is the case, then some organ primordia in Ufo Ap3 or Ufo 
Pi flowers will perceive no class A/B/C activity and will develop as leaves.

4.3.2 Ufo Pi and Ufo Ap3 double mutant phenotypes

Figures 4.2A and 4.2D show the phenotypes of mutations in the genes *PI* and *AP3.* 
Pi-1 and Ap3-1 phenotypes involve transformation of petal to sepal and stamen to carpel 

Double mutants, and mutants homozygous at one locus and heterozygous at the 
other from both the *ufo-1 X pi-1* and *ufo-1 X ap3-1* F$_2$ populations had almost 
indistinguishable phenotypes but were distinct from the single mutant parents. We will 
refer to this as the double mutant phenotype. The double mutant phenotype could be 
considered additive with respect to Ufo, Pi, and Ap3 single mutant phenotypes. The 
apparent dominance of *ufo* in a class B mutant background and similarly of class B 
mutations in a *ufo* mutant background suggests that, in flowers where class B activity has
already been weakened by homozygosity at one of these loci, the remaining class B activity is limiting and dosage dependant. Thus further reductions in class B activity, by removing a functional allele at the other locus, causes a change at the phenotypic level that would not have been observed under wild type, non-limiting conditions.

Organs in double mutant flowers were assigned to whorls based on their position, and ambiguous cases were not used in organ type analysis. Double mutant flowers consisted of sepal, carpel, sepal/carpel mosaic, and filamentous organs. Unlike Pi and Ap3 single mutant phenotypes, second and third whorl organs exhibited both sepal and carpel features, consistent with the appearance of reproductive tissue in the perianth whorls, and perianth tissue in the reproductive whorls of Ufo-1 single mutant flowers. Gynoecial structures from double mutants often produce little or no ovary tissue. Typical Ufo-1 Pi-1 flowers are shown in Figures 4.2B and 4.2C. Table 4.1 lists the frequencies of second and third whorl homeotic transformations from 142 Ufo-1 Ap3-1 double mutants grown at 16°C. Flowers from this population (Figures 4.2E and 4.2F) have slightly weaker transformations than those in Ufo-1 Pi-1.

### Table 4.1 Frequency of Organ Types Appearing in Second and Third Whorls of Ufo-1 Ap3-1 Double Mutant Flowers

<table>
<thead>
<tr>
<th>Whorl</th>
<th>Sepals</th>
<th>Filamentous Organs With Trichomes</th>
<th>Filamentous Organs Without Trichomes</th>
<th>Filamentous Organs Fused to Gynoecium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second</td>
<td>49</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Third</td>
<td>60</td>
<td>93</td>
<td>22</td>
<td>175</td>
</tr>
</tbody>
</table>

*Numbers of each organ type appearing in 142 flowers grown at 16°C; organs in ambiguous positions were not scored.*
The appearance of reproductive tissue in the second whorl and perianth tissue in the third whorl of Ufo-1 Ap3-1 and Ufo-1 Pi-1 flowers suggests that the *ufo-1* mutation causes aberrant expression of class C organ identity function. In addition, the lack of leafy organs in these double mutant flowers indicates that class A and C activity continue to occupy exclusive and exhaustive domains within Ufo flowers.

4.4 Ufo exhibits changes in *FLO10* activity

*UFO* appears to be an enhancer of class B gene expression in the second and third whorls. Another gene, *FLO10* is known to be a class B inhibitor. In Flo10-1 flowers, carpels are replaced by stamens and stamen-carpel mosaic organs (Figure 4.1D). Whorls 1, 2, and 3 are initiated normally in organ position, type, and number (Schultz, et al., 1991; Bowman, et al., 1992). This phenotype is believed to result from *AP3* and *PI* expression in the fourth whorl. If *UFO* is a general regulator of class B activity, then double mutants between *ufo* and *flo10* should appear similar to those between *pi* or *ap3* and *flo10*, generating the pattern sepal, sepal, carpel, carpel. However, if *UFO* acts to regulate class B activity only in the second and third whorls, the phenotype of Ufo Flo10 double mutant might be additive, exhibiting Ufo-like transformations in the second and third whorls, and Flo10-like transformations in the fourth whorl.

Ufo-1 Flo10-1 double mutant flowers (Figure 4.1E) have characteristics of both single mutants; however the phenotype is not properly described as additive. First whorl organs are sepals, however it is difficult to determine the position of other organs due to a significant organ loss from the second, third and/or fourth whorls. Few apparent second
whorl organs appear (eight organs in 39 flowers) but all show the range of transformations seen in second whorl organs of Ufo-1. Organs that are apparently in lateral third whorl positions usually mature to free-standing stamens, whereas apparent medial third whorl organs are stamens or mosaics of stamen and carpel tissue. These fuse with other carpelloid organs, possibly originating from fourth whorl positions. Thus, as expected, fewer fourth whorl stamens are produced in double mutant flowers than in Flo10-1 alone. However, the appearance of third whorl stamens and staminoid organs in all double mutant flowers was surprising because Ufo-1 does not generate staminoid structures until the latest flowers. Thus the flo10-1 mutation appears to suppress the Ufo-1 phenotype in the third whorl.

This result can be explained in the following way: It has been proposed that FLO10 is a regulator of cell divisions; cells that express both FLO10 and class B genes are unable to divide, thus a boundary is formed between whorls three and four. In the absence of FLO10, division of class B-expressing cells is allowed (Sakai et al., 1995). Presumably, in Ufo mutants, class B activity is lower, or class B-expressing cells are more dispersed. Thus in combination, you might expect the reduction in overall class B expression by ufo to largely suppress fourth whorl staminoid features, however the release, by flo10, of class B-expressing cells from mitotic arrest in the inner whorls should result in an increased number of such cells in the third/fourth whorl region, resulting in an increased number of staminoid structures over that seen in Ufo alone. However, it is not clear from this model why Ufo Flo10 double mutants exhibit a high degree of organ loss.
4.5 *UFO* acts in the FLIP mechanism

4.5.1 General FLIP mutant features

Several aspects of the Ufo-1 phenotype suggest that mutant plants are defective in initiating the floral program. To study this effect further, double mutants were constructed using the *ufo-1* allele and mutant alleles of two FLIP genes, *LFY* (*lfy-1* and *lfy-2*) and *API* (*apl-1*).

Double mutants between FLIP genes have been well characterized (Bowman et al., 1993; Schultz and Haughn, 1993). In general, FLIP double mutants exhibit synergistic phenotypic interactions, usually resulting in plants that produce no floral shoots at all. If *UFO* represents a novel FLIP gene, *ufo* may be expected to act in a similar manner.

4.5.2 Ufo Lfy double mutant phenotype

Strong *LFY* mutant alleles (e.g., *lfy-1*) cause all lateral shoots to develop as coflorescences. All primary and lateral shoot apical meristems terminate development with the production of carpel-like structures. All other floral organs are absent. Weaker alleles (eg. *lfy-2*) generate more coflorescence-like structures than does the wild type; however, some 'flowers' are generated from which new lateral shoots may arise. Lfy-2 floral organs show a wide range of transformations and are often mosaics of two or more organ types (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel, et al., 1992).
Because the ufo-1 mutation exhibits primarily a floral phenotype in CL, it was anticipated that mutations at the LFY locus should be partially or completely epistatic to ufo-1. Indeed, the F² populations of both ufo-1 X lfy-1 and ufo-1 X lfy-2 crosses exhibit wild type, Ufo-1, and a range of Leafy-like phenotypic classes in a ratio indicating that the double mutant phenotypes are Lfy-like.

To assist in identifying double mutant individuals, Lfy-2 plants were crossed with the linked marker line ufo-1 dis-2 that was generated during the mapping of UFO. Lfy-2 Ufo-1 Dis-2 plants from the F² of this cross consistently generate fewer floral-like nodes and terminate apical growth earlier than Lfy-2 alone suggesting that, as expected, ufo-1 enhances the weak Leafy-2 phenotype.

4.5.3 Ufo Ap1 double mutant phenotype

Strong mutant alleles of API such as ap1-1 generate flowers that exhibit a lack of perianth structures and the replacement of these with bracts from which axillary 'floral' shoots arise (Figure 4.1F) (Irish and Sussex, 1990; Bowman, et al., 1993; Schultz and Haughn, 1993).

Ufo-1 Ap1-1 double mutants in CL produce a phenotype similar to that of a strong Lfy mutant. A rosette of seven to ten leaves is formed followed by up to four coflorescence nodes subtended by normal bracts. All other lateral meristems develop as coflorescence-like shoots, occasionally subtended by filamentous structures. Each lateral shoot consists of up to 11 nodes (mean 6.46 nodes per coflorescence) with a leaf or leaf-carpel mosaic organ and axillary meristem at each node (Figure 4.1G). The amount of
carpel tissue within these mosaic organs increases acropetally, and terminal organs frequently fuse. As in both Ufo-1 and Lfy plants, all apical and lateral shoots terminate in a pistil-like structure. Elongation does not occur in some of the more apical lateral shoots, giving them a flowerlike appearance although neither sepal, petal nor stamen tissues can be seen (Figure 4.1H). Ontogenic analysis revealed that primordia on lateral shoots have a spiral phyllotaxy similar to that of an inflorescence (Figure 4.1I). No lateral shoots produce organs in an obviously whorled pattern. Thus the inflorescence structure of the double mutant is similar to that seen in strong Leafy mutants; however, unlike Leafy, only the most basal lateral shoots are subtended by normal bracts.

The double mutant interactions between UFO and FLIP gene mutations are consistent with the hypothesis that UFO is required for proper floral initiation. ufo enhances, in a synergistic manner, the flower to coflorescence transformations in both Ap1 and weak Lfy phenotypes. Double mutant plants are unable to generate any floral structures even in the most apical nodes, suggesting that ufo mutations further weaken the ability of the FLIP mutant plant to initiate the floral program.

In their analysis, Levin and Meyerowitz examined the phenotype of several additional double mutant combinations: Ufo FL54, Ufo Leunig, Ufo Clavata1 and Ufo Clavatav3 (Levin and Meyerowitz, 1995). In each case, the double mutant plants exhibited an enhanced number of floral nodes from which no lateral shoot was produced, but which retained a filamentous structure, interpreted as an abnormal leaf or bract. These filamentous structures appear to be similar to those described in this analysis which appear in Ufo-1 under short days (Figure 3.2C). Since Ufo, FL54, Clv1, and Clv3 mutants are all able to generate flower-like structures, the synergistic interactions between ufo and these
mutants was interpreted as additional evidence that *UFO* is involved in the floral initiation process.

4.6 Ufo plants remain responsive to the COPS signal

*UFO* appears to be required for proper floral initiation and acts like a FLIP gene. One of the features of FLIP mutants is an acropetal decrease in phenotypic severity. Thus the floral program in FLIP mutants appears to remain responsive to COPS inhibition even in the absence of a fully functional FLIP. Ufo plants also exhibit an acropetal decrease in phenotypic severity. Mutations in the *TFL1* gene cause a reduction in the overall levels of COPS activity, thus causing phase switches at earlier nodes. To examine if the floral program in Ufo plants is COPS sensitive, double mutants were constructed between *ufo-1* and *tfll-14*.

4.6.1 Ufo Tfl double mutant phenotype

Mutations at the *TFL1* locus cause replacement of coflorescences with flowers and early termination of growth when the shoot apex itself becomes a floral meristem (Shannon and Meeks Wagner, 1991; Alvarez, et al., 1992).

Ufo-1 Tfl1-14 plants have an inflorescence structure similar to that of Tfl1-14 alone; however, all flowers including the terminal flower exhibit transformations similar to those seen in Ufo-1 single mutant individuals. Notably, organ types in Ufo-1 Tfl1-14
double mutant flowers are typical of those observed in the latest flowers of Ufo-1 single mutant plants.

Although flowers from Ufo Tfl double mutants exhibit weaker organ type transformations than in Ufo alone, this is consistent with what is expected if the floral program is inhibited by COPS activity. In Ufo Tfl plants floral development is altered by the absence of UFO product. However, unlike Ufo single mutant individuals where COPS activity is high throughout the plant, Ufo Tfl double mutants have low levels of COPS and the floral program in double mutants is more active; even in early nodes. Thus the floral program appears to be responsive to COPS activity in Ufo mutants.

4.7 Discussion of double mutant analyses

Three major hypotheses were tested through double mutant analysis: 1) Ufo flowers have reduced or altered class B function, 2) Ufo plants exhibit abnormal class A/C expression domains, and 3) UFO acts in the floral initiation process.

Ufo Ag, Ufo Ap2-1, and Ufo Ap2-6 double mutants all exhibited features indicating that the ufo mutation causes a reduced class B gene function. Thus one role of the UFO gene is to act as an activator of class B genes, or regulate their domains of expression.

Ufo Ap3 and Ufo Pi double mutants exhibited features consistent with an aberrant establishment of the class A/C boundary, but showed that this boundary is likely maintained, as in wild type, through antagonistic interactions between class A and C genes. Thus UFO appears to be required to regulate the domains of expression for class A
and C organ identity functions.

Finally, *UFO* exhibits all of the features shared by the FLIP genes, and enhances the phenotype of other FLIP mutations, consistent with its designation as a new member of the FLIP gene class. Ufo exhibits its strongest FLIP enhancement in combination with *ap1* and *lfy*, but little or no enhancement of FLIP features in combination with *ap2*. This indicates that *UFO* may have more functional redundancy with the FLIP activities of *LFY* or *AP1* than with *AP2* or that *ap2* is epistatic to *ufo* with respect to its FLIP functions.

The appearance of completely normal coflorescences in positions apical to early flowers in the Ufo single mutant indicates that *UFO* has unique role in interpretation of the COPS signal, and the transduction of that signal to the other FLIP genes.

Thus *UFO* appears to have two roles: 1) *UFO* is involved in floral initiation, and 2) *UFO* is involved in establishing boundaries of gene activity within the flower. In the Ufo mutant phenotype these two roles are strikingly distinct.

Unlike other FLIP mutations that dramatically alter flower morphology, *UFO* lateral shoots are distinctly coflorescence or flowerlike. This feature of the *UFO* phenotype makes it possible to use the technique of *in situ* hybridization to unambiguously compare transcript patterns of FLIP and organ identity genes between Ufo and wild type structures.
5. IN SITU HYBRIDIZATION OF ORGAN IDENTITY GENES TO Ufo

5.1 Introduction

The model for flower development predicts that each organ identity gene should be expressed in the particular domain over which it has control. This is supported by in situ hybridization studies, which revealed that the class A gene APETALA1 transcript was present in the first and second whors (Figure 5.1G) (Mandel et al., 1992; Bowman et al., 1993), APETALA3 and PISTILLATA were expressed in whors two and three (Figure 5.1D) (Jack et al., 1992; Goto and Meyerowitz, 1994), and AGAMOUS transcript was present in whors three and four (Figure 5.1A) (Drews et al., 1991; Yanofsky et al., 1990). AP2 transcript is reported to be detected throughout the plant (Jofuku et al., 1994).

In Ufo flowers it appears that the organ identity genes are expressed aberrantly, showing effects on both level and domain. Thus it is important to determine at what level this regulation is taking place. In situ hybridization was performed using antisense RNA probes against several FLIP and organ identity genes on wild type and mutant tissue. If UFO exerts its regulatory effects at the level of transcription it should be possible to detect changes in the levels and/or pattern of gene transcripts using this technique. In addition, the UFO gene itself (see chapter 6) was subcloned into a plasmid that allowed production of UFO antisense RNA. In the following sections data obtained from these in situ hybridization studies is discussed in the context of the both the Ufo phenotype, and the expected results based on single and double mutant analyses. Stages of flower development are from Smyth (Smyth, et al, 1990) and are briefly described in Table 5.1.
Table 5.1. Stages of Flower Development (from Smythe et al., 1990)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1:</td>
<td>Floral primordium is distinguishable on the flank of the apical meristem.</td>
</tr>
<tr>
<td>Stage 2:</td>
<td>Dome of floral primordium is clearly separate from the apex; no organ primordia are visible.</td>
</tr>
<tr>
<td>Stage 3:</td>
<td>Sepal primordia appear on the flanks of the floral apex.</td>
</tr>
<tr>
<td>Stage 4:</td>
<td>Sepals overlie the floral primordium.</td>
</tr>
<tr>
<td>Stage 5:</td>
<td>Stamen and petal primordia appear.</td>
</tr>
<tr>
<td>Stage 6:</td>
<td>Sepals overlie petal and stamen primordia.</td>
</tr>
<tr>
<td>Stage 7:</td>
<td>Stamens have distinct anther and filament sections.</td>
</tr>
<tr>
<td>Stage 8:</td>
<td>Stamens develop locules.</td>
</tr>
</tbody>
</table>

5.2 Transcript patterns of organ identity genes in Ufo plants

Data from single and double mutant analyses indicate that class B gene expression should be reduced in Ufo flowers. DIG labelled antisense RNA was generated against coding sequences from $PI$ and $AP3$ and used to probe wild type, Ufo-1, Ufo-3, Ufo-4, and Ufo-5 tissues. It is anticipated that transcript levels should be lower in mutant tissue than in wild type tissue, and might be variable from position to position within the flower, consistent with the variability in organ type within $UFO$ flowers.

Wild type transcript patterns and levels were consistent with those in previously published studies (Jack et al., 1992; Goto and Meyerowitz, 1994). Both $PI$ and $AP3$ transcript become detectable in late stage 2 flowers, $AP3$ transcript is limited to the region of the second and third whorls, while $PI$ occupies second, third, and fourth whorl positions. As primordia move into stage 3 and beyond, cells are detectable in the centre of the dome that are free of $PI$ transcript (Figure 5.1A). As organ primordia become visible
(stages 4 and beyond), transcript is detected only in cells occupying the second and third whorls. Late in development transcript from both genes remain detectable at high levels throughout second and third whorl organs (Figure 5.1A).

Ufo flowers probed with PI and AP3 antisense showed no differences in early expression pattern or level compared to wild type. Transcript was detected in late stage 2 floral primordia and was lower in the central region of the dome (Figure 5.1B). As flowers matured transcript was detected only in second and third whorl primordia. As organ primordia developed further, transcript often appeared to be restricted to just the outer cell layers of the organ (Figure 5.1C). Although wild type second and third whorl organs did not exhibit this late transcript patterning, it is difficult to compare Ufo and wild type directly since these organs in Ufo are likely to have developed as 'filaments' that have no obvious wild type counterpart. These results were consistent for all alleles of ufo examined.

Other studies (Levin and Meyerowitz, 1995) presented evidence that class B gene transcripts are significantly reduced in stage 2 and 3 Ufo flowers, but return to normal levels later in development. Although this may be more consistent with the Ufo phenotype, I could find no evidence to support this claim. Several possibilities could account for this discrepancy: 1) The techniques used by our two groups differ in the method of detection (radioactive versus non-radioactive probes); It is possible that detection of radioactive probes for in situ studies are less sensitive than non-radioactive detection methods. Thus the amount of transcript may be below the level of detection
Figure 5.1: In situ hybridization of FLIP and organ identity genes to wild type and Ufo inflorescences. Dark stained regions indicate detection of transcript by DIG-labelled antisense RNA. Numbers above floral primordia indicate the stage of flower development. When possible, the shoot apex (a) is indicated.

(A) Hybridization of *PI* antisense RNA to wild type flowers. A stage 3 flower is shown in which levels of *PI* are beginning decrease in the central regions (arrow) (140X).

(B) Hybridization of *PI* antisense RNA to Ufo-5 flowers. Stage 2 and 3 flowers are shown. The stage 3 flower shows levels of transcript (arrow) almost identical to those of the stage 3 flower in (A) (140X).

(C) Hybridization of *PI* antisense RNA to a stage 7-8 Ufo-5 flower. Arrows indicate cells on the interior of the organ that do not exhibit hybridization (140X).

(D) Hybridization of *AG* antisense RNA to a wild type inflorescence (160X).

(E) Hybridization of *AG* antisense RNA to a Ufo-1 inflorescence (130X). Compare to (D).

(F) Hybridization of *AG* antisense RNA to a late Ufo-1 shoot apex. Arrows indicate presence of transcript on the flanks of the shoot apical meristem. It is likely that this meristem is about to terminate by generating carpel-like structures (170X).

(G) Hybridization of *API* antisense RNA to a wild type inflorescence (130X).

(H) Hybridization of *API* antisense to a Ufo-1 inflorescence (250X).

(I) Hybridization of *API* antisense to a stage 3 flower. Compare the pattern of staining with that of wild type stage 4 (G) (170X).

(J) Hybridization of *AP2* antisense to a wild type inflorescence. Arrows show detection of transcript in the putative first and second whorl regions of the stage 2 flower (180X).

(K) Hybridization of *AP2* antisense to a Ufo-3 inflorescence (140X).

(L) Hybridization of *AP2* antisense to a Ufo-3 inflorescence. Arrows point to detection of transcript only in the first and second whorls (140X).
using their radioactive methods, while we were able to detect these small amounts of
transcript through non-radioactive techniques. Since in situ hybridization is not reliably
quantitative, we might not be able to determine if we are detecting lower than normal
levels of transcript 2) Growing conditions between the two groups are not identical;
growth conditions are known to significantly affect phenotypes and thus may account for
the differences observed. 3) Either group may have generated erroneous results.
However, immunolocalization using antibodies against class B protein indicates that little
or no class B product exists in Ufo flowers (Levin and Meyerowitz, 1995), thus we believe
Ufo may be regulating these genes post-transcriptionally.

Data from single and double mutant analyses suggest that the expression domain of
class C organ identity function is not consistently limited to third and fourth whorl tissues.
DIG labelled antisense RNA probe was generated from the coding region of the AG gene
and used to probe wild type, Ufo-1, and Ufo-3 tissues. It was anticipated that AG
transcript might be detected in some second whorl primordia, and be absent from some
third whorl primordia in Ufo flowers.

Wild type tissue probed with AG showed transcript patterns similar to those
described in the literature (Drews et al., 1991; Yanofsky et al., 1990). AG transcript is
visible in late stage 2 flowers in the centre of the floral primordium. As organ primordia
become visible transcript is apparent in primordia from the third and fourth whorls,
consistent with the whorls that are affected by the ag mutation (Figure 5.1D).

Ufo flowers probed for AG transcript exhibited no differences in transcript pattern
or level when compared to wild type. AG transcript became visible in stage 2 primordia,
and in more mature flowers was apparent in third and fourth whorl floral organs. Because
Ufo flowers generate aberrant numbers of organs there is some difficulty in determining the whorl designation of an organ from a paraffin section; however no clear cases were observed where $AG$ transcript was absent from a third whorl or present in a second whorl primordium (Figure 5.1E). As the organs matured, transcript remained detectable in similar patterns to that seen in wild type mature organs. Unlike wild type, $AG$ transcript in Ufo mutants is present in late inflorescence apices (Figure 5.1F). This is consistent with the development of carpel-like organs during termination of the Ufo inflorescences.

5.3 Transcript patterns of FLIP genes in Ufo plants

Single and double mutant analyses suggest that $UFO$ plays a role in the regulation of floral initiation, and interacts synergistically with the FLIP genes $LFY$ and $AP1$. As described earlier, FLIP genes appear to be independantly activated early in presumptive floral primordia, and may interact to enhance each others expression. Moreover, FLIP genes such as $AP1$ and $AP2$ seem to have a significant role in regulation of the $AGAMOUS$ expression domain. Thus it is important to investigate the transcript patterns of FLIP genes in Ufo flowers to determine if $ufo$ mutations alter the level or pattern of FLIP gene expression.

DIG-labelled antisense RNA was generated from the coding regions of $AP1$, $AP2$, and $LFY$ clones, and was used to probe sections of wild type and Ufo-1 tissues. Several possible results were anticipated: 1) $UFO$ seems to be involved in floral initiation, particularly given the striking appearance of ectopic coflorescences in SD Ufo flowers. It is, therefore, possible that $UFO$ represents a regulator of FLIP genes. If this were the
case, then FLIP gene transcript levels might be reduced in Ufo flowers. 2) FLIP genes appear to be activated independently of one another. If UFO represents a novel FLIP gene, the absence of UFO product may have no effect on the transcript patterns of other FLIP genes. 3) Since API has been shown to regulate AG transcript domains, and the Ufo phenotype suggests that AG activity is altered in Ufo flowers, it is possible that mutations in UFO may affect the domain of API transcription.

Transcript patterns of LFY and API in wild type tissue were consistent with those reported by other groups (Mandel et al., 1992; Weigel et al., 1992), while my observation of AP2 transcript patterns differ from those published. LFY and API transcripts are detected in early stage 1 flowers throughout the primordium, with API being stronger along the adaxial edge of the primordium (Figure 5.1G). LFY transcript is maintained throughout the floral meristem, appearing in all organ primordia as they arise. API transcript is maintained only in the perianth whorls, and is detectable throughout late first and second whorl organs (Figure 5.1G). AP2 transcript appears prior to the formation of any organ primordia, and is later present in early first and second whorl primordia, but absent from the central dome of the floral meristem (Figure 5.1J), thus the early pattern of AP2 transcript is consistent with the organs that are affected by mutant alleles at this locus. Transcript appears in third and fourth whorl primordia as they are initiated, however it is quickly lost from third whorl organs. Late Ap2 flowers contain transcript in petals and carpels only.

No differences were observed in the transcript patterns of LFY, API (Figure 5.1H, I) or AP2 (Figure 5.1K, L) in Ufo flowers when compared to wild type. Thus UFO does not appear to be a direct activator or regulator of the domain of expression of any of
these genes, although these results are not inconsistent with the designation of UFO as a FLIP gene member.

6. MOLECULAR ANALYSIS OF THE UFO GENE

6.1 Introduction

Cloning of the UFO locus was undertaken in collaboration with Dr. Enrico Coen and colleagues at the John Innes Institute, UK. A gene involved in floral organ identity in Antirrhinum majus had been identified (SEPALOIDEA - Schwarz-Sommer et al., 1990; FIMBRIATA Simon, et al., 1994) and cloned (Simon et al., 1994). The mutant phenotype of this gene had several similarities to the Ufo phenotype, and thus it was proposed by Dr. Zsuzsanna Schwarz-Sommer (Max Planck Institute, Koln) that the two genes might be homologous. The method by which the UFO locus was cloned, and the relative contributions of each group are described in the following sections. All work done in the John Innes institute has been published (Ingram, et al., 1995).

6.2 Identification of the putative FIM homologue in Arabidopsis

In preparation for RFLP analysis, I crossed ufo-1 (Col-2) plants to wild type plants of the Ler ecotype. 67 F₂ plants, including both Ufo and wild type phenotypes, were allowed to self-fertilize. The resulting F₃ families were examined for segregation of the
Ufo phenotype, and in this way the genotype of each F₂ individual was inferred. I then extracted DNA from each F₃ family.

Dr. Coen's group probed *Arabidopsis* genomic λ-libraries with a fragment from the *FIM* open reading frame. Three classes of λ clones showed strong hybridization, and these were used to probe southern blots of wild type Col-2 X Ler DNA digested with various enzymes. One clone showed a restriction fragment length polymorphism (RFLP) with the enzyme HindIII (2.5kb; 2.7kb), a second showed an RFLP with CfoI (8kb; 5kb) and a the third was polymorphic when cut with HaeIII (1kb; 2kb).

I digested DNA from the F₃ families previously generated with each of these three enzymes, and blots of this DNA were sent to Dr. Coen's group. These blots were probed with each of the potential *UFO* homologues to determine if any exhibited co-segregation between the RFLP and the *ufo* *UFO* alleles. In addition, each of the λ clones was screened a second time using a DNA fragment from a gene located approximately 1kb from the *FIM* locus. One of the classes of λ clones identified an RFLP that co-segregated with the *ufo* allele (no recombinant chromosomes in 67 individuals/134 chromosomes), and cross hybridized with the probe from the adjacent gene, indicating that the linkage observed between *Antirrhinum* genes had been conserved in *Arabidopsis* and that this class of clones represented the *FIM* homologue. This class consisted of three overlapping clones: λJAM2001, λJAM2002, λJAM2003.

### 6.3 Complementation of the Ufo phenotype by the *FIM* homologue

Based on the known *FIM* sequence Dr. Coen's group selected a 6 kilobase (kb)
fragment that was believed to contain all of the putative open reading frame plus approximately 4kb of upstream sequence, but no coding sequences from the downstream gene. They subcloned this fragment into a binary vector (pJAM199) for use in Agrobacterium transformation. This vector also carried a selectable marker for Kanamycin resistance (Kan-r), which would be used to distinguish transformants from non transformed, Kanamycin sensitive (Kan-s) plants.

I had previously generated a line of ufo-1 carrying a linked marker dis2, which causes a trichome phenotype (linkage of approximately 0.5 map units). I provided this line to Dr. Coen for complementation analysis. Complementation was attempted in two ways: (1) Agrobacterium was used to introduce the wild type FIM homologue into plants heterozygous for ufo-1 and dis2. Kan-r plants, representing the successful transformants, were selected. These were selfed resulting in a line that segregated both Kan-s:Kan-r and wild-type:Dis2 in a 3:1 ratio. Among the Kan-r plants, they observed Dis-2 individuals that had a novel floral phenotype less extreme than Ufo-1. (2) Agrobacterium was used to transform the FIM homologue into wild type Ler plants. Lines homozygous for Kan-r were selected and crossed to Ufo-1 Dis2 plants. They self-fertilized the Kan-r F1 population and Dis2 Kan-r F2 plants were examined. In all cases, these plants exhibited a phenotype that was less extreme than that of Ufo-1, some lines generated ufo-1 homozygous plants that were almost completely wild type. These results suggest that the FIM homologue represents the UFO gene.

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6.4 Sequence analysis of the UFO gene and mutant alleles

Sequence analysis was done entirely by Dr. Coen and his colleagues. The sequence contained a single open reading frame of 1326 base pairs (bp) that has the potential to code for a 442 amino acid (aa) protein of 49 kilodaltons. Base and aa sequences were compared to those in sequence databases but no significant homology was found with any previously cloned loci other than the FIM gene itself.

Homology between UFO and FIM was extensive, with an overall aa similarity of 72% with 60% identity. Conservation was particularly strong in two regions of the protein; between aa41 and 88 (77% identity), and between aa212 and 374 (78% identity). These two regions did not exhibit significant similarity to any proteins in the sequence databases.

Three mutant alleles were examined for changes in base sequence: ufo-1, ufo-3, and ufo-5. The Ufo-1 phenotype results from a single G to A nucleotide change at base 855 that creates a stop codon leading to a truncated protein of 285 aa. Ufo-3 results from a single base deletion at base 178, resulting in a truncated protein of 108 amino acids. Ufo-5 results from the creation of a stop codon resulting in a truncated protein 326aa in length.

6.5 Timing and distribution of the UFO transcript in wild type development

All in situ hybridization analysis was done by the author. The plasmid pJAT170, containing 530bp of the UFO coding region, was used to generate DIG labelled antisense
RNA. Sections of wild type inflorescences and flowers were probed for the presence of

*UFO* transcript.

*UFO* transcript is detected in the earliest stage of floral development at the point where the floral primordium is barely discernable from the shoot apex (early stage 1) (Figure 6.1A). This timing is similar to the appearance of FLIP gene transcripts such as *LFY* and *API*. At this stage, transcript was most prevalent in the abaxial-central cells of the newly initiated floral meristem, and extended down through three to four cell layers. I will refer to this as the 'early' *UFO* transcript pattern. Transcript is not detected in any apical meristems, or other vegetative or inflorescence tissues (Ingram, et al., 1995).

The pattern of transcript changes dramatically over the following two stages of flower development. In stage 2 flowers, transcript is distributed in a ring approximately three to four cells wide surrounding an area in the centre of the floral primordium that is free of transcript (Figure 6.1C)(Ingram et al., 1995). Probing alternate serial sections with *UFO* or *AG* antisense transcripts indicates that the inner edge of this ring may be directly adjacent to the outer edge of the domain of *AG* transcript for at least the early stages (late stage 2 to stage 3) of flower development (Figure 6.1D,E). This observation is consistent with a similar observation made in *Antirrhinum* using *FIM* and *PLENA*, the *Antirrhinum* homologue of *AG*. By the time sepal primordia appear (stage 3), transcript is detectable in a thin ring of cells just inside the sepal whorl, possibly occupying the region destined to become the second whorl (Figure 6.1B)(Simon et al., 1994). A short time later, transcript is retained in only a few of these cells, located at the abaxial base of each petal primordium (Figure 6.1C). Transcript remains in these cells until stage 10 or later. I will refer to these as the 'late' *UFO* transcript patterns (Ingram et al., 1995).
6.6 Transcript patterns of *UFO* in FLIP and organ identity mutants

To determine if the *UFO* gene is regulated by any of the known FLIP genes, *UFO* transcript patterns were observed in the FLIP gene mutants Lfy-1, Lfy-2, Ap1, Ap1/Cal, and Ap2. A similar analysis was done in the organ identity gene mutants Pi, and Ag.

6.6.1 *UFO* transcript patterns in Lfy.

Phenotypic analysis of Ufo Lfy double mutants indicated that lfy mutations were almost completely epistatic to ufo mutations, suggesting that *UFO* activity may be significantly reduced in Lfy mutant flowers. However, *in situ* hybridization of *UFO* antisense transcript to inflorescences of Lfy-1 and Lfy-2 plants indicated that *UFO* transcript was abundant in most or all lateral shoots, although these shoots were destined to produce coflorescences. Transcript was located in the abaxial-central regions of the shoot meristems in a manner that may be analogous to the 'early' *UFO* transcript pattern seen in wild type stage 1 meristems (Figure 6.2A). No meristems were observed that exhibited 'late' *UFO* transcript patterns, although *UFO* transcript was distributed in variable patterns in older shoot apices (Figure 6.2B).


Ap2 is believed to have a role in both floral initiation (FLIP) and floral organ
identity. Double mutant analysis of Ufo Ap2 flowers indicated that removing UFO activity from an Ap2 flower results in loss of any organ types associated with class B activity. However, this additive phenotype is quite subtle since class B activity is already very low in Ap2 flowers due to ectopic AG activity. Loss of UFO activity does not appear to significantly enhance the FLIP mutant features of Ap2 mutants.

UFO antisense transcript was hybridized to sections of Ap2-6 flowers. Most flowers contained no detectable UFO transcript (Figure 6.2C), however some young floral primordia contained very low levels of UFO transcript (Figure 6.2D). The rare cases where hybridization occurred exhibited patterns that did not correlate well with the discreet 'early' or 'late' patterns associated with wild type UFO transcripts (Figure 6.2E). Transcript was never detected in any floral primordia beyond stage 3 (Figure 6.2E).

6.6.3 UFO transcript patterns in Ap1 and Ap1 Cal.

Phenotypic analysis of Ufo Ap1 double mutant plants indicated that loss of UFO product greatly enhanced flower to coflorescence transformations of Ap1 mutants. This indicates that UFO is required for the floral structures in Ap1 plants.

Surprisingly, hybridizations of UFO antisense transcript to sections of Ap1-1 and Ap1-10 flowers and inflorescences suggest that UFO is almost completely absent from these plants (Figure 6.2F). Close examination of some early Ap1-10 floral meristems revealed a few cells that exhibited low levels of UFO transcript (Figure 6.2G). As in Ap2 primordia, there were rarely enough cells exhibiting transcript to determine with certainty
**Figure 6.1:** In situ hybridization of *UFO* to wild type tissue. Numbers indicate the approximate stage of floral development of the associated flower primordium.

(A) Hybridization of *UFO* antisense transcript to wild type inflorescence. Transcript is detected in a broad region in the stage 1 primordium (390X).

(B) Hybridization of *UFO* antisense transcript to wild type stage 3 flower. Transcript is limited to a few cells just interior to the developing sepals (400X).

(C) Hybridization of *UFO* antisense transcript to wild type tissue. Arrows indicate a ring of transcript in the stage 2 flower. In the stage 6 flower transcript is detected at the abaxial base of each petal primordium (230X).

(D) and (E) Hybridization of *AG* and *UFO* antisense transcript to sequential sections through a stage 2 flower primordium. Arrows point to approximately the same position on each section. There appears to be no overlap between *UFO* and *AG* transcript domains (350X).
any spatial distribution pattern.

Hybridization of *UFO* antisense to sections of Ap1 Cal double mutant plants revealed high levels of *UFO* transcript in many 'Cauliflower' meristems. Transcript was distributed in a pattern similar to the 'early' *UFO* transcript pattern in wild type flowers (Figure 6.2H). No meristems were observed that exhibited the typical 'late' *UFO* transcript patterns. Transcript did not seem to appear in all shoot primordia; it is likely that it is present only in higher-order meristems, many of which are destined to form some flower-like structures. This would be consistent with the reports of increasing amounts of *API* and *LFY* expression in higher order Ap1 Cal meristems (Bowman, et. al., 1993).

6.6.4 *UFO* transcript patterns in Ag.

The Ag phenotype consists of a nested series of [sepal, petal, petal]. In wild type flowers, *UFO* transcript is associated with second whorl organs, being located at the abaxial base of each petal.

*UFO* antisense transcript was hybridized to sections of Ag flowers. Typical 'early' transcript patterns are observed (Figure 6.3A), and transcript appears in a ring in the stage 2 floral meristem. In older flowers, however, transcript was detected at the abaxial base of many floral organs (Figure 6.3B,C). Close observation of serial sections from these older flowers suggests that *UFO* is absent from certain whorls of organs, however it was not possible to determine with certainty which whorls lack transcript. It is plausible that transcript is associated with the two petal whorls of each nested flower, but absent from sepal whorls, although other possibilities exist.
6.6.5 *UFO* transcript patterns in Pi

*UFO* transcript in Pi floral primordia exhibits the same general 'early' and 'late' patterns as seen in wild type. Transcript is first apparent in stage 1 primordia, and quickly forms a ring in stage 2 meristems (Figure 6.3D). Late in development, transcript becomes limited to four positions corresponding to the positions of the second whorl organs (Figure 6.3E), although it is worth noting that, unlike wild type, these organs will develop as sepals in the Pi mutant; thus petal development is not necessary for the late *UFO* transcript pattern. Closer examination of some primordia suggests that, although the overall pattern of transcript is similar to that of wild type, the domain of transcript may be uneven in some primordia. Figure 6.3F shows a stage 2 primordium in which the domain of *UFO* transcript is unusually wide (approximately 4-5 cells) on one side of the meristem compared to the other (approximately 2 cells). Since, in wild type flowers, this is the stage at which *UFO* transcript becomes limited to petal primordia, it is difficult to determine if this observation is significant; however *UFO* transcript in wild type flowers never appeared in such a broad domain.

6.6.6. *UFO* transcript patterns in Ufo

To determine if *UFO* is autoregulatory, *UFO* antisense transcript was hybridized to sections of strong Ufo-3 mutant flowers and inflorescences. In all cases, transcript appeared in the 'early' and 'late' patterns typical of wild type *UFO* transcript (Figure 6.3G,H). This indicates that *UFO* transcription is not dependant on *UFO* activity.
Figure 6.2: In situ hybridization of *UFO* to FLIP mutant inflorescences. Numbers indicate the approximate developmental stage of the associated primordium. Where possible the shoot apex (a) is indicated.

(A) Hybridization of *UFO* antisense RNA to *Lfy*-1 inflorescence. A lateral shoot (s) is shown. Bracts (b) are visible subtending additional lateral shoots (120X).

(B) Hybridization of *UFO* antisense RNA to *Lfy*-1 lateral shoots (s) (170X).

(C) Hybridization of *UFO* antisense RNA to an *Ap2*-6 inflorescence. No transcript was detected (150X).

(D) Hybridization of *UFO* antisense RNA to an *Ap2*-6 inflorescence. No transcript is detected in the stage 1 floral primordium, however some weak hybridization is apparent in the stage 2 primordium. Contrast on this figure has been significantly strengthened to make the signal more apparent (250X).

(E) Hybridization of *UFO* antisense RNA to an *Ap2*-6 inflorescence. Uneven hybridization is apparent in the stage 1 primordium. No transcript is apparent in the stage 4 primordium (180X).

(F) Hybridization of *UFO* antisense RNA to an *Ap1*-1 inflorescence. Almost no signal is detected in this inflorescence (150X).

(G) Hybridization of *UFO* antisense RNA to an *Ap1*-1 inflorescence. Weak and disperse signal is seen in the stage 2 and 3 primordia. Contrast has been adjusted to more clearly reveal signal (200X).

(H) Hybridization of *UFO* antisense RNA to an *Ap1*-1 *Cal* double mutant inflorescence. Transcript is clearly visible in many of the inflorescence meristems (arrows) (100X).
Figure 6.3: In situ hybridization of UFO to organ identity mutants and Ufo. Numbers indicate approximate developmental stage of the associated flower primordium. Where possible the shoot apex (a) is indicated.

(A) Hybridization of UFO antisense RNA to stage 3 Ag-1 flower. Transcript is detected in a pattern similar to a wild type flower of similar age. Arrow shows transcript is present on one side of the primordium, indicating that the 'ring' of transcript has already begun to become more restricted. Earlier and later sections show transcript in other regions of the same primordium (not shown) (140X).

(B) and (C) Hybridization of UFO antisense RNA to older Ag-1 flowers. Arrows indicate detection of transcript in inner whorls of nested flowers.

(D) Hybridization of UFO antisense RNA to a Pi inflorescence. Transcript is present in a typical ring pattern in the stage 2 flower primordium shown (250X).

(E) Hybridization of UFO antisense RNA to a Pi inflorescence. As in wild type, transcript is associated with petal primordium in the stage 3 and 4 flowers shown (120X).

(F) Hybridization of UFO antisense RNA to a stage 2 Pi flower. Arrow points to an unusually broad region of transcript. The more narrow region on the opposing side of the same primordium is more typical of UFO transcript patterns (compare with (I) - 140X).

(G) and (H) Hybridization of UFO antisense RNA to Ufo-3 inflorescences. Transcript is detected in patterns similar to that observed in wild type. Arrows point to narrow regions of transcript at the abaxial base of petal primordia (G - 250X; H - 400X).

(I) Hybridization of UFO antisense RNA to wild type stage 2 primordium. Arrows indicate the narrow regions of transcript typical of wild type flowers (260X).

(J) and (K) Hybridization of UFO antisense RNA to Ufo-3 flowers. Arrows indicate unusually large domains of UFO transcript in these stage 3 flowers (J - 190X, K - 290X).
Although the overall pattern of transcript distribution appears similar to wild type, the number of cells exhibiting transcript seems to be greater in Ufo primordia. Figure 6.3I shows a typical wild type primordium at late stage 2. *UFO* transcript at this stage is limited to a ring approximately 1-2 cells wide. Figure 6.3J and K show hybridization to slightly older Ufo-3 primordia. Although in wild type transcript distribution becomes even more limited at this stage, the number of cells exhibiting transcript in Ufo-3 is notably larger than the wild type primordium shown. Thus, although *UFO* does not depend on *UFO* activity for its continued transcription, the correct distribution of *UFO* transcript does appear to depend on *UFO* activity.

### 6.7 Discussion of molecular analyses

The *UFO* gene has been cloned based on its homology to the *FIMBRIATA* gene of *Antirrhinum*. The *UFO* gene appears to contain a single open reading frame coding for a putative 442 amino acid protein. The evidence that the entire coding sequence has been identified is as follows: (1) The homologous *FIM* gene also contains a single open reading frame. (2) The cloned sequences were sufficient to fully complement the Ufo phenotype, and these contained only the single open reading frame, in addition to extensive upstream sequences. No mapping of transcript ends has been attempted to date.

Two regions of *UFO* appear to be critical for function of the *UFO* product based on the high degree of conservation in these two regions between *FIM* and *UFO*. However, neither of these regions shows a high correspondence to any sequences in the sequence database. Neither does the overall sequence of either gene give any indication for putative
function.

The proposed early and late roles of UFO are consistent with in the wild type UFO transcript distribution. UFO is activated sufficiently early and is quite broadly distributed in the stage 1 floral primordium, in a pattern consistent with its proposed role in the FLIP mechanism. Later, UFO transcript appears in a ‘ring-like’ pattern consistent with a role in regulating the domain of at least one floral organ identity gene, AG.

UFO and FIM have similar patterns of transcript distribution. Both genes are expressed early in flower initiation, and later appear in a ring with an inner boundary corresponding to the outer boundary of AG (PLE) transcript. This pattern becomes more restricted at later stages of development, however UFO transcript is present only at the abaxial base of each petal, while FIM transcript appears as a ring around each petal primordium. In addition, a small region of FIM activity is visible between the third and fourth whorls of older wild type floral meristems, which is not observed in Arabidopsis (Simon et al., 1994).

That UFO (FIM) and AG (PLE) transcript domains share a boundary is intriguing in light of the apparent effects of ufo mutations on AG expression, although ufo mutations have no conspicuous effect on AG transcript distribution. It is plausible to suggest that the regulation of AG by UFO occurs post-transcriptionally; possibly at the level of protein diffusion, or through regulating cell division patterns within the floral meristem to prevent mixing of cells with differing fates.

Throughout early flower development, UFO transcript patterns bear little correlation to its apparent regulation of class B activity; the UFO domain only partially overlaps the class B domain. However, UFO does share characteristics, both at the
phenotypic and molecular level, with the FLIP gene class. It is known that FLIP gene mutations exhibit their strongest effects on the expression of Class B genes, although their expression patterns are not closely correlated with the class B domain. Thus it is possible that *UFO* exerts its effect on class B activity early in flower initiation by weakening the activity of FLIP.

Several unexpected results were observed during the analysis of *UFO* transcript patterns in FLIP and organ identity mutants. The apparent lack of cells expressing *UFO* in Ap1 mutants is surprising given the strong interaction of these two mutations at the phenotypic level. It is possible that the FLIP in Ap1 plants is sufficiently weakened that even a slight loss of additional FLIP activity results in a complete loss of floral functions. A similar lack of *UFO* transcript was observed in Ap2 flowers. Both Ap1 and Ap2 have a role in regulating *AG* activity, and in both mutants *AG* activity may be observed in perianth whorls. It is possible that this *AG* activity is repressing transcription of *UFO*. This is supported by the phenotype of Ap1 Ap2 Ag triple mutants, which have a phenotype similar to Ap1 Cal (Bowman, et al., 1993); if *AG* activity is reduced in the Ap1 Cal double mutant that could explain why *UFO* transcript is detected in the double mutant, but not in the Ap1 single mutant. Alternately, it is possible that, in these mutants, cells expressing *UFO* do not divide and become scattered throughout the floral meristem as the surrounding cells continue dividing. This might account for the more 'dispersed' patterns of cells in which *UFO* could be detected.
7. ROLE OF UFO IN FLOWER INITIATION AND DEVELOPMENT

7.1 UFO is Involved in the Floral Initiation Process

Most data from ufo-1 single and double mutant analyses support the interpretation that UFO has a role in initiating floral development in lateral shoots (FLIP). The wide array of homeotic transformations observed in Ufo-1 flowers have also been observed in flowerlike structures from weak Lfy and Ap1 mutants (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel, et al., 1992; Bowman, et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). Like FLIP mutant individuals, Ufo-1 plants exhibit an enhanced number of coflorescence-like lateral shoots in SD beyond that seen in the wild type. Bracts or bract-like structures subtend many 'floral' shoots in both Leafy and Ufo-1 and are particularly pronounced in the most apical nodes. Ufo shows an acropetal decrease in phenotypic severity, enhances flower to coflorescence transformations in combination with Ap1 or Lfy alleles, exhibits numerous transformations consistent with reduction of class B function, and exhibits termination of apical meristem growth accompanied by production of carpelloid structures. Finally, UFO transcript appears in the earliest stages of floral development at approximately the same time as other FLIP gene transcripts, and is independant of other FLIP genes for its transcription. Each of these features is common among FLIP genes (Irish and Sussex, 1990; Huala and Sussex, 1992; Weigel, et al., 1992; Schultz and Haughn, 1993). These similarities are compelling evidence that UFO plays a role in the FLIP process. A diagram is shown in Figure 7.1 that indicates the relative role of UFO in the FLIP compared to other genes involved in floral initiation. UFO is placed
below AP1 but above AP2 due to the stronger effect of ufo mutations on inflorescence structure compared to Ap2, and its stronger enhancement of the Ap1 phenotype.

Each FLIP gene examined has had a unique phenotype, having some features shared with other FLIP genes and other features specific to that locus. Ufo also has a unique phenotype and unique features. Unlike the Lfy and Ap1 phenotypes, the transition from coflorescence to flower production is pronounced such that any given shoot is conspicuously flowerlike or coflorescence-like. Moreover, in SD conditions, Ufo plants produce coflorescences apical to flowers, and floral nodes from which no shoot is produced. This phenotype implies that the fate of lateral shoots in Arabidopsis is determined independently of adjacent lateral shoots. The fact that only flowerlike structures are generated in the most apical nodes of Ufo-1 indicates that, in these nodes, the fate determination mechanism is operating correctly. Thus, it appears that ufo-1 interferes with the ability of FLIP to correctly interpret COPS signal at near-threshold levels. The appearance of 'empty' flowers suggests that, even if a floral fate is chosen, the commitment to this fate is weak or unstable in Ufo-1. Other aspects of floral morphology are altered in Ufo-1, and these are discussed in section 7.2 and 7.3.

Because AP2 might be considered a FLIP gene (Irish and Sussex, 1990, Bowman, et al., 1993, Schultz and Haughn, 1993), it is interesting that the Ufo-1 Ap2-1 and Ufo-1 Ap2-6 phenotypes are not synergistic with respect to their floral initiation phenotypes, as was observed in the Ufo-1 Ap1-1 double mutant phenotype. There are at least three possible explanations: (1) Both UFO and AP2 play sufficiently minor roles in the FLIP under CL that no enhancement of FLIP mutant features was apparent in the double mutant, (2) UFO and AP2 have few redundant activities in the FLIP, or (3) The alleles chosen were
'weak' enough that synergistic effects were not apparent; although it is known that ufo-1 is the result of a nonsense mutation (see section 6.4), this mutation appears near the end of the putative open reading frame, and thus the protein product may have some activity. In situ hybridization analysis indicates that the level of UFO transcript in Ap2 plants is very low, indicating that one or both of explanations (1) and (2) may be true.

Molecular and genetic analyses have shown that individual FLIP genes are largely independant of each other for their transcription (Weigel et al., 1992; Bowman, et al., 1993; Gustafson-Brown, et al., 1994; Jofuku, et al., 1994; Weigel and Meyerowitz, 1994). Thus, the wild-type transcript patterns of LFY, API, and AP2 in Ufo plants are not inconsistent with the designation of UFO as a FLIP gene. Moreover, the high levels of UFO transcript in Lfy and Apl Cal mutant flowers indicates that UFO is similarly independant of FLIP gene activity.

7.2 UFO regulates developmental processes within the flower

Figure 7.1 indicates the relative roles of UFO and other FLIP genes in the regulation of developmental processes within the flower. It should be noted that there appears to be significant overlap between the roles of API and AP2 in flower-specific activities, compared to that of UFO and API/AP2, suggesting that these two groups of genes control different subsets of the functions governed by LFY. This may account, in part, for the stronger phenotype of Ufo Apl double mutants when compared to Ap2 Apl double mutants (Irish and Sussex, 1990; Schultz and Haughn, 1993).

Several lines of evidence indicate that UFO is needed to establish domains of
Figure 7.1. Role of *UFO* in floral initiation and development.
The vertical axis represents the degree of participation of each gene in the FLIP, as indicated by the shaded gradient. The horizontal axis indicates several functions required for proper floral development. The genes *LFY*, *AP1*, *CAL*, *UFO*, and *AP2* are placed in horizontal boxes spanning the functions that appear to be regulated by each gene. Graded lines indicate that the given gene plays a lesser role in the associated floral function compared to other genes.
function of the floral organ identity genes. The appearance of reproductive tissues in perianth whorls and perianth tissue in reproductive whorls of Ufo single mutant and Ufo Pi or Ufo Ap3 double mutants suggests that class C organ identity activity is variable from organ to organ in the first, second and third whorls. Thus, UFO appears to have a role in regulating the domain of class C activity.

The FLIP genes AP2, API, and LFY are also involved in the regulation of class C function, and many organ type transformations observed in Ufo are similar to those observed in Ap2 and Ap1 individuals. For example, carpelloid first whorl organs are common in Ap2-6 and petal-stamen mosaic organs are observed in Ap1 (Schultz and Haughn, 1993; Bowman, et al., 1993). However, unlike API and AP2 where mutations affect primarily first and second whorl organ type, UFO has its strongest effect on organ type in the second and third whorls.

The whorls occupied by class B gene function (whorls 2 and 3) are those that exhibit the greatest effect in Ufo-1, and many of the organ type transformations in Ufo and double mutant combinations can be accounted for by reduction of class B gene function. However, there is significant evidence that UFO does not represent a novel class B gene. No known mutant alleles of class B genes affect the expression of class A or C gene function, nor do any known class B genes affect floral initiation. Further, no ufo allele affects the class B domain to the same extent as mutations in PI or AP3.

Consistent with the apparent reduction of class B activity in Ufo flowers, ufo mutations are able to suppress the production of stamens in the fourth whorl of Flo10 flowers. It is surprising, however, that flo10 is able to partially suppress the effects of ufo mutations in the third whorl. As discussed earlier (see section 4.4), this may be accounted
for through aberrant regulation of cell divisions by flo10, ufo, or both.

Molecular results further enhanced the interpretation of these genetic analyses. Despite the phenotypic evidence supporting the suggestion that class B gene function is reduced and class C gene function varies spatially, in situ hybridization results revealed little difference in the amount or pattern of PI, AP3, or AG transcripts in Ufo flowers when compared to the wild type. Several possibilities might account for the apparent discrepancy between the Ufo phenotype and results from in situ hybridization. First, the regulation of the organ identity genes by UFO could be post-transcriptional. Second, the variability of the Ufo phenotype indicates that class B and C gene function is affected only in some flowers and only in particular regions within a given flower. In addition, organ type transformations are seldom complete in Ufo flowers, thus the effect of UFO on organ identity gene transcription may be subtle. Because it is difficult to quantitate steady state transcript levels with in situ hybridization results such changes might not be easily detectable. Third, the Ufo phenotype might not depend on the specific class B and C genes used as probes for the in situ hybridization analyses. Because there are undoubtedly additional organ identity genes, it is possible that UFO exerts its effects through other, as yet unidentified class B and C genes.

In situ hybridization results show that the domains of AG and UFO transcript in stage 2 flowers are, at least briefly, adjacent and non-overlapping, consistent with the suggestion that UFO regulates the class C domain. However, the lack of UFO transcript in Ap2 flowers suggests that ectopic AG expression is able to suppress UFO transcription. The domain of UFO expression in AG mutants is also altered; UFO appears at the base of many organs in the nested flowers. This could be interpreted as a 'spreading' of the UFO
domain in the absence of $AG$; alternately, $UFO$ may be activated independantly and reiteratively in each whorl of the Ag flower. Thus $UFO$ and $AG$ may be mutually regulatory.

$UFO$ transcript patterns in mutants with reduced class B function indicate that $UFO$ appears to depend, at least in part, on class B activity for its proper spatial regulation, although not for its activation. Domains of $UFO$ transcript in both Pi and Ufo-3 plants appeared to be larger than that of wild type. Since $UFO$ transcript in wild type is detected only in the abaxial base of each petal (the outer border of the class B domain) in late stage 3 flowers, these results suggest that class B expression may be antagonistic to $UFO$ activity.

Together, these molecular analyses lead to a surprising view of $UFO$ regulation in which $UFO$ is activated early in the FLIP process, is required to regulate class B and C expression, but is suppressed by both class B and C activities.

7.3 $UFO$ influences other aspects of inflorescence and flower development

The production of carpels in terminating inflorescence shoot apices is a feature shared by many FLIP mutant phenotypes (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel, et al., 1992; Schultz and Haughn, 1993). Ufo plants are unique, however, in that primary meristem termination occurs much earlier than in other FLIP mutations. In situ hybridization analysis of terminating Ufo inflorescences shows that this event is correlated with the appearance of $AG$ transcript in the apex of Ufo inflorescences, although it appears to be excluded from the meristematic region. Because no known FLIP gene has
been shown to be transcribed in the apex of inflorescence shoots, it is not clear how FLIP genes regulate \textit{AG} transcription in this region. It has been suggested from studies with male sterile mutants, and plants from which flowers have been surgically removed, that normal flowers are involved in signalling the shoot apex to remain indeterminate (Hensel, et al., 1994). However, \textit{UFO} must have an additional role in this process because the absence of the \textit{UFO} gene product causes apical termination earlier than expected. Some sterile plants also generate carpelloid first whorl organs and bracts subtending the most apical floral nodes (Hensel et al., 1994), suggesting that some features commonly observed in FLIP mutant phenotypes might be due to sterility.

Changes in organ number are common in mutations affecting floral initiation (Schultz and Haughn, 1993). The primary difference between \textit{ufo} and other FLIP mutations is that FLIP mutants such as Ap2 and Ap1 exhibit only reductions in organ number, whereas Ufo flowers may also generate additional organs in the first, third, and fourth whorls, and moreover, additional whorls of organs under SD conditions. Little is known about the regulation of organ and whorl number. Investigation of the Clavata mutant phenotypes (Leyser and Furner, 1992, Clarke, et al., 1993) has revealed that increases in meristem size are associated with increased organ number. Measurements of floral meristems showed that Ufo-1 floral meristems were significantly larger than those of wild type, but the differences were not significant in Ufo-3 plants. Because additional organs are rare and a maximum of one additional organ has been observed in a single flower, it is possible that the changes in meristem size are too small to be measured accurately. The unusual growth of Ufo Ag floral meristems further supports the suggestion that \textit{UFO} has a role in regulating meristem size or identity.
CONCLUDING REMARKS

We have shown that the *UFO* gene is involved in at least two processes. First, it is required for floral initiation, in combination with the other FLIP gene members. Second, it has a role in the regulation of all classes of floral organ identity gene activity, particularly the expression of class B genes. The transcript patterns of *UFO* are largely consistent with these proposed functions. *UFO* is not unique in having multiple roles throughout plant development. It is becoming apparent that each FLIP gene differs in the degree to which it participates in the FLIP decision and in the particular subset of downstream developmental processes regulated by that gene (Schultz and Haughn, 1993; Bowman, et al., 1993; Shannon and Meeks-Wagner, 1993; Okamuro, et al., 1993).

*UFO* might act in several ways: Although organ identity gene transcript distribution appeared unaffected by *ufo* mutations, *UFO* may act post transcriptionally to regulate the activity of these genes. *UFO* may act to regulate movement of organ identity gene products (or other regulatory molecules) between meristematic cells. Alternately, the loss of early FLIP activity in Ufo flowers may result in later pleiotropic effects on the domains of organ identity gene activity. Finally, *UFO* might regulate domains of activity by regulating the number/position of cells expressing particular organ identity gene activities.
FURTHER INVESTIGATIONS

It is clear that \textit{UFO} is involved in spatial regulation of organ identity genes, however there is a discrepancy between the observed phenotypic changes, and the transcript patterns of the organ identity genes in Ufo mutants. It is known that certain organ identity genes are regulated post-transcriptionally (Jack et al., 1994), thus this investigation would benefit from \textit{in situ} hybridization studies using antibodies against individual organ identity genes.

A second confusing aspect of the Ufo phenotype is the phenotypic effect of \textit{ufo} mutations on organs in which \textit{ufo} is never conspicuously expressed (e.g. the organ-type changes in the third whorl and changes in fourth whorl organ number). It is possible that these are pleiotropic effects of early disruption in the FLIP, or alternately they may indicate that \textit{UFO} acts in a non-cell-autonomous manner. The ability to test cell-autonomy has been reasonably well developed in the Antirrhinum system through utilization of unstable transposon-induced mutants. Thus it may be possible to investigate whether the product of the \textit{UFO} homologue, \textit{FIM}, is able to diffuse between cells of the floral meristem.

In addition, it has recently been suggested that some class B organ identity gene patterning may take place at the level of mitotic control (Sakai et al., 1995) through the \textit{FLO10} gene. Flo10 mutants also exhibit phenotypic effects in whorls that do not express \textit{FLO10}, and further, \textit{FLO10} is expressed along the inner border of class B gene activity, in a pattern that may be analogous to the expression of \textit{UFO} along the outer class B border. Thus it is tempting to speculate that \textit{UFO} may act in a similar manner to \textit{FLO10}. Such an
analysis would require a careful study of cell division patterns in wild type and Ufo mutants.

Finally, the availability of the *UFO* clone enables construction of transgenic plants that express the *UFO* product ectopically, either in a general manner under the control of the 35S promoter, or in a directed manner through use of promoters from other cloned organ identity genes. Such studies could significantly clarify the role of *UFO* in both the FLIP and later flower development. For example, it has recently been shown that over-expression of the FLIP gene *API* is able to compensate for the lack of a second FLIP gene, *LFY*. It would be interesting to perform a similar analysis through introduction of *UFO* into Lfy or Ap1 plants. Further, over-expression of *UFO* in wild type flowers may clarify the function of *UFO* in regulation of the organ identity genes.
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