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Separable roles of *UFO* during floral development revealed by conditional restoration of gene function

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SUMMARY

The *UNUSUAL FLORAL ORGANS* (*UFO*) gene is required for several aspects of floral development in *Arabidopsis* including specification of organ identity in the second and third whorls and the proper pattern of primordium initiation in the inner three whorls. *UFO* is expressed in a dynamic pattern during the early phases of flower development. Here we dissect the role of *UFO* by ubiquitously expressing it in *ufo* loss-of-function flowers at different developmental stages and for various durations using an ethanol-inducible expression system. The previously known functions of *UFO* could be separated and related to its expression at specific stages of development. We show that a 24- to 48-hour period of *UFO* expression from floral stage 2, before any floral organs are visible, is sufficient to restore normal petal and stamen development. The earliest requirement for *UFO* is during stage 2, when the endogenous *UFO* gene is transiently expressed in the centre of the wild-type flower and is required to specify the initiation patterns of petal, stamen and carpel primordia. Petal and stamen identity is determined during stages 2 or 3, when *UFO* is normally expressed in the presumptive second and third whorl. Although endogenous *UFO* expression is absent from the stamen whorl from stage 4 onwards, stamen identity can be restored by *UFO* activation up to stage 6. We also observed floral phenotypes not observed in loss-of-function or constitutive gain-of-function backgrounds, revealing additional roles of *UFO* in outgrowth of petal primordia.

Key words: *Arabidopsis thaliana*, Flower development, Conditional expression, *UFO*

INTRODUCTION

A general model proposes that regulatory genes expressed in specific domains of the developing flower set up the early patterning and trigger later specific events of growth and differentiation (Lohmann and Weigel, 2002; Weigel and Meyerowitz, 1994). Although many of these regulators can act at multiple developmental stages, it is difficult to define their role at any particular stage. Temperature-sensitive alleles have provided useful insights into temporal requirements (Bowman et al., 1989; Zachgo et al., 1995), but unfortunately few such alleles exist for most plant genes. Conditional expression provides an equivalent means to address the general problem of varying temporal requirements of any given gene function for which a loss-of-function allele is available. In this paper, we use this approach to reveal the temporal requirement of *UNUSUAL FLORAL ORGANS* (*UFO*) during flower development.

UFO is involved in several aspects of flower development (Ingram et al., 1995; Lee et al., 1997; Levin et al., 1998; Samach et al., 1999; Wilkinson and Haughn, 1995). First, *UFO* interacts with *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Mandel et al., 1992; Weigel et al., 1992) to specify the floral identity of the meristem (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Secondly, *UFO* plays a role in floral organ identity control. Organ identities at given positions of the floral meristem are specified by a combinatorial action of three classes of genes (Bowman et al., 1991; Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). According to this ABC model, sepal identity is conferred by the action of A class genes, represented by *APETALA1* and *APETALA2* (*AP1* and *AP2*) (Jofuku et al., 1994; Mandel et al., 1992). Class A genes in combination with the class B genes *APETALA3* (*AP3*) and *PISTILATA* (*PI*) (Goto and Meyerowitz, 1994; Jack et al., 1992) confer petal identity. Stamen identity results from the combined action of class B and class C genes, such as *AGAMOUS* (*AG*) (Yanofsky et al., 1990). Finally class C genes alone confer carpel development. Each class of identity genes acts in two adjacent whorls of the floral meristem, class A in whorls 1 and 2, class B in whorls 2 and 3 and class C in whorls 3 and 4. Recently, it has been shown that the function of the ABC genes also require *SEPALLATA1*, *2* or *3*, three functionally redundant genes (Jack, 2001; Pelaz et al., 2000). *UFO* has a major role in promoting B function as evidenced by the lack of normal petals and stamens in *ufo* loss-of function mutants and the supernumerary petals and stamens observed in lines overexpressing *UFO* (Lee et al., 1997; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995).

Furthermore, second and third whorl defects in *ufo* mutants can be rescued by ectopically expressing B-class genes (Krizek and Meyerowitz, 1996).

In addition to perturbed identities during floral development, defects in the growth of organ primordia have been reported for the *ufo* mutants. Therefore, a further role of *UFO* in coordination of organ identity gene expression and the growth patterns has been suggested (Ingram et al., 1997; Levin and Meyerowitz, 1995; Samach et al., 1999; Wilkinson and Haughn, 1995).

In agreement with its complex developmental role, the expression pattern of the *UFO* in the flower is highly dynamic (Lee et al., 1997; Samach et al., 1999). Early on, *UFO* is expressed in the central dome of the floral meristem, after which it becomes progressively restricted to the presumptive whorls 2 and 3 and, finally, to the base of the petals. The predicted UFO protein has given some hints to its potential function. UFO is the orthologue of FIMBRIATA from *Antirrhinum majus* and contains an F-box motif conserved in plant, mammalian and yeast proteins, which interacts physically with SKP1-related proteins (del Pozo and Estelle, 2000; Ingram et al., 1995; Ingram et al., 1997; Samach et al., 1999; Simon et al., 1994; Zhao et al., 1999; Zhao et al., 2001). F-box proteins associate with a large protein complex called SCF, that has an E3 ubiquitin ligase activity and targets specific proteins to degradation through the ubiquitin/proteasome pathway. The specificity of the SCF complex is conferred by the interaction between the F-box protein and the target protein. Targets for UFO-containing SCF complex have not been identified to date, but in other systems the known target proteins fall mainly in two classes: cell cycle regulators and transcription factors (Patton et al., 1998).

We have examined the changing roles of *UFO* during flower development by expressing *UFO* ubiquitously in *ufo*-2 mutant flowers, at different developmental stages and for various durations, using an ethanol inducible expression system. In this way, the previously known functions of *UFO* could be temporally separated and related to its expression at specific stages of development. Early *UFO* expression is required during stage 2 for normal patterning of the primordia in the three innermost whorls. Activation of the B genes can be mediated by *UFO* expression during floral stage 2 or 3 but petals and stamens differ in their period of competence to respond to *UFO*: stamen primordia have a wider window than petal primordia. We also show that growth of the petals requires a short pulse of *UFO* during early stages of their development, indicating that *UFO* has an additional role in petal outgrowth. Finally, our data suggest a role for *UFO* in the regulation of the size of the third whorl, possibly through interaction with *SUPERMAN*.

MATERIALS AND METHODS

Construction of plasmids and plant transformation

A *UFO* fragment containing the entire ORF was reconstructed by joining the inserts of pJAM195 and pJAM196 (Ingram et al., 1995) at the *Hin*dIII site. The ATG initiation site of the *UFO* ORF was site-mutagenised to introduce a *Nco*I site, creating pJAM180. A *Nco*I fragment of pJAM180 containing the full *UFO* ORF and 743 bp downstream of the STOP codon was polished using Klenow and inserted into the *Sma*I site of pL4 (Syngenta Ltd, Jeolotts Hill, UK) between the p*AlcA* promoter and the terminator of the 35S CaMV, generating pL4-UFO. An *Xba*I fragment containing the alcA-UFO expression cassette was excised from pL4-UFO and ligated into the *Hin*dIII restriction site of the binary vector pSRN/AGS (Roslan et al., 2001) containing the 35S::ALCR cassette. The resulting binary vector was electroporated into *Agrobacterium* strain GV3101 and *Arabidopsis* was transformed as described previously (Clough and Bent, 1998). Transgenics were selected in the presence of 50 mg/l kanamycin.

Plant growth and ethanol induction

Seeds were pretreated in water at 4°C for 2 days to ensure synchronous germination and sown on soil in 6 × 6 × 6 cm pots (5 plants per pot). Plants were grown in growth chambers under long day conditions (8 hours dark at 17°C, 16 hours light at 20°C for 1 hour, 23°C for 14 hours and 20°C for 1 hour, at 70% humidity). Alternatively, in order to synchronise the flowering time, plants were initially grown for 21-25 days under short-day conditions (16 hours dark at 16°C, 8 hours light at 20°C, 70% humidity) before transfer to long-day conditions. Ethanol induction was achieved by irrigating each pot daily for 5 days with 3 ml of 1% (v/v) ethanol and covering the plants with a 10-cm high transparent lid during the time of induction. Vapour induction was achieved by placing open 500 μl microtubes filled with 95% (v/v) ethanol into every alternate pot for 8 hours every day and covering the plants with a lid, then the ethanol tubes were removed and the lid opened for the remaining 16 hours. Observations were on the main inflorescence.

Transcription analysis by RT-PCR

Total RNA were extracted from 5 apices of induced or non-induced *35S::UFO*$_{ind}$ plants using TRIzol reagent (Life Technologies) according to the supplier's instructions, including a centrifugation before chloroform extraction to minimise DNA contamination. After DNase treatment (1 unit DNase Amp Grad; Life Technologies) for 20 minutes at room temperature, 2.5 μg of total RNA was reverse transcribed for 50 minutes at 37°C in a final volume of 20 μl in the presence of 250 ng oligo(dT) primers, 5 mM $MgCl_2$, 1 mM of dNTPs and 50 Units of M-MLV (Eurobio, Les Ulis, France) in the reaction buffer provided. Reactions were stopped by heat inactivation and 80 μl of H_2O were added. 5 μl of the reverse transcription reaction were used for PCR amplification. The primers pUFO2 (CTTCAGGATCATCAGGAGGGTTAG) and pUFONRI (TCTTGAATTCAAAGCGGCCGCAACAGACTCCAGGAAATGG-AAGTGTT) gave an 872 bp PCR product for the endogenous and transgene cDNA and the contaminating genomic DNA. Absence of contaminating genomic DNA was confirmed by the absence of PCR products in a preparation lacking reverse transcriptase. The primers pAPT-1 (TCCCAGAATCGCTAAGATTGCC) and pAPT-2 (CCTTTCCCTTAAGCTCTG) amplified the adenine phosphorybosiltransferase cDNA (Moffat et al., 1994) and were used as a quantitative control. For quantification, 14 cycles of PCR were conducted (30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C) followed by radioactive hybridisation and quantification of the radioactive signal using a BAS-1500 Fujifilm phosphoimager.

The distinction between the transcripts generated by the endogenous mutated *ufo*-2 gene and the wild-type *UFO* transgene was based on the *Afl*III polymorphism described by Lee et al. (Lee et al., 1997). The *Afl*III restriction site was absent from the PCR product generated with the pUFO2 and pUFONRI primers on wild-type template whereas *Afl*III digestion of the PCR product obtained from mutated template gave rise to 550 bp and 322 bp bands.

In situ hybridisation

In situ hybridisation was performed as described previously (Laufs et al., 1998). DIG-labelled *UFO* probe was generated by in vitro transcription of the *Eco*RI linearised plasmid pJAM170 (Ingram et al., 1995).

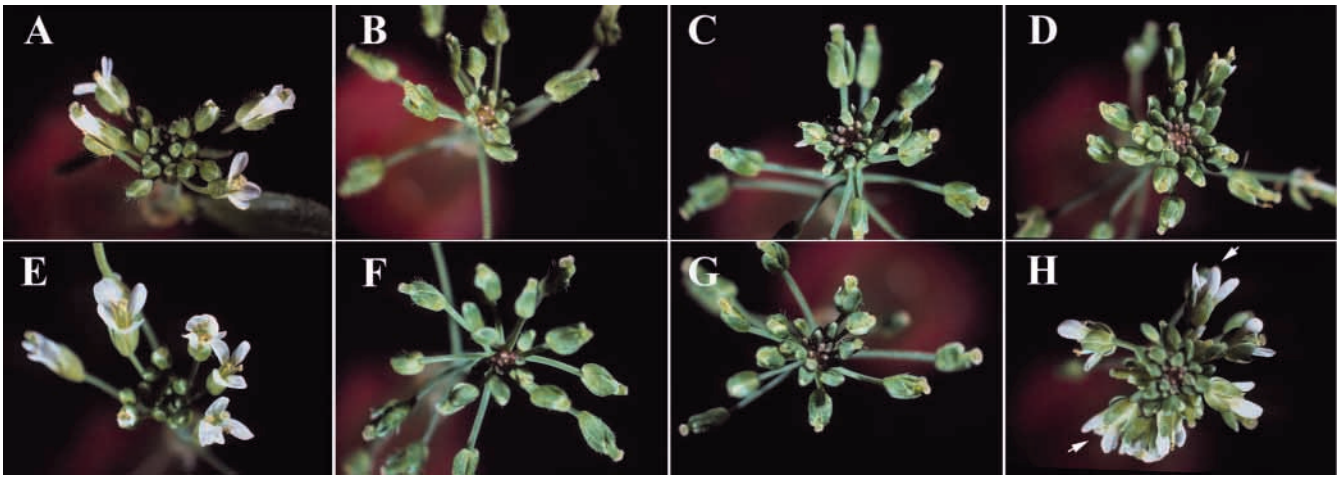

Fig. 1. Ethanol-induced restoration of the *ufo-2* mutant. Plants were not induced (A-D) or induced for 5 days by ethanol after 3 weeks culture under Long Days. (E-H). The apices were observed 18 days after the beginning of the treatment. No difference in floral morphology was observed between wild type (A,E) and *ufo-2* (B,F) lines whether treated or not treated with ethanol. Flowers of *ufo-2 35S::uidA*$_{ind}$ transgenics carrying an inducible GUS construct (C,G) were not modified by ethanol induction. (D,H) In *ufo-2 35S::UFO*$_{ind}$ ethanol-induced expression of *UFO* restored petals and stamens and wild-type flowers (arrows) could be observed (H).

Scanning electron microscopy

Apices were analysed by low-temperature scanning electron microscopy as described previously (Traas et al., 1995). Alternatively, flowers were fixed overnight in 3% (v/v) glutaraldehyde in 25 mM sodium phosphate buffer pH 7.0, dehydrated in a graded ethanol series at 4°C and critical-point dried in liquid carbon dioxide. Samples were dissected, mounted, carbon-gold shadowed and observed in a 525M Philips scanning electron microscope.

RESULTS

Ethanol-induced restoration of *ufo* mutants

To investigate the different functions of *UFO* during the floral development of *Arabidopsis*, we used the ethanol-inducible system derived from the filamentous fungus *Aspergillus nidulans*, shown to function in different plant species including *Arabidopsis* (Caddick et al., 1998; Roslan et al., 2001; Salter et al., 1998; Sweetman et al., 2002). This system is based on two components: the transcription factor ALCR, whose activity depends on the presence of low levels of ethanol, and the promoter p*AlcA* that is activated by ALCR. In our constructs, *ALCR* is expressed ubiquitously under the control of the CaMV 35S promoter. The *UFO* gene and, as a control the *uidA* gene coding for GUS, were placed under the control of the inducible promoter p*AlcA*. The inducible constructs, hereafter called *35S::UFO*$_{ind}$ and *35S::uidA*$_{ind}$, were introduced into wild-type *Arabidopsis*, ecotype Landsberg *erecta*.

Ethanol induction was achieved either by irrigating the plants daily with 1% (v/v) ethanol for 5 days or by exposing them to ethanol vapour in confined conditions for various durations (see Materials and Methods). *35S::uidA*$_{ind}$ lines showed ethanol-dependent GUS staining as previously reported (Roslan et al., 2001) (data not shown). Induction of *35S::UFO*$_{ind}$ in the wild-type background led to flowers with slightly petaloid sepals, an increased number of petals and, to a lesser extent, stamens (results not shown).

The constructs were introduced into mutants carrying the strong *ufo-2* allele to generate *ufo-2 35S::UFO*$_{ind}$ and *ufo-2 35S::uidA*$_{ind}$ lines, homozygous for both the mutation and the transgene. Flowers of *ufo-2* mutants showed defects in all four whorls, with the second and third whorls being the most affected (compare Fig. 1A,B, Fig. 2A,B). Whorls 1 and 4 showed variation in the number and size of the organs, with occasionally 3 or 5 sepals of irregular size and 2-4 carpels with occasional fusion defects in the distal part of the pistil. Normal petals and stamens were missing and replaced by sepal-like organs, filaments, carpeloid or staminoid structures. In addition, the number and position of the organs were abnormal, with occasional united growth of organs in or between whorls.

Ufo-2 35S::UFO$_{ind}$ flowers showed restoration of petals and stamens approximately 16-18 days after the beginning of a 5-day-long treatment with 1% (v/v) ethanol irrigation, starting after 3 weeks culture under long days (Fig. 1H). Wild-type flowers with 4 sepals, 4 petals, 4-6 stamens and a pistil with two carpels could be observed in almost all the inflorescences (Fig. 1H, Fig. 2G). Restoration was dependent on the ethanol treatment and was not observed in either non-transformed or GUS-transformed *ufo-2* plants (Fig. 1). A gradient of phenotypic restoration was observed above and below fully restored flowers. This suggested that the temporal regulation of *UFO* expression was critical to its function and that this might be amenable to experimental dissection.

Transient *UFO* expression can restore normal development of whorls 2 and 3

We first addressed the question of the minimal duration of *UFO* expression required for normal petal and stamen development. For this, *ufo-2 35S::UFO*$_{ind}$ plants were subjected to ethanol treatments for various periods. We used vapour induction in preference to induction by irrigation in order to have better control of the timing of induction. Table 1 shows the mean number of flowers with normal petals and stamens per main apex following ethanol induction. A single 8-hour ethanol pulse was sufficient to restore normal petal and stamen development

Fig. 2. Floral phenotypes in wild type, *ufo-2* and different phases of phenotypic restoration in *ufo-2 35S::UFO*$_{ind}$. (A) Wild-type flower, with 4 sepals (not visible), 4 petals, 6 stamens (only five visible) and a pistil with 2 carpels. (B) *ufo-2* flower with no petals and stamens The four outer sepals have been removed. Two filaments (arrows), 1 out of 2 staminoid sepal (arrow), 1 carpeloid structure (asterisk), and 2 out of 4 inner sepals are visible. The pistil contains two fused carpels. (C) Type I *ufo-2 35S::UFO*$_{ind}$ flower with 4 first whorl sepals, 1 petaloid sepal (arrowhead), 1 staminoid petal (arrow), 1 petal, 8 stamens and a pistil with 3 unfused carpels. (D) Type I *ufo-2 35S::UFO*$_{ind}$ flower with 5 first whorl sepals, 1 sepaloid petal (arrow), 1 petal, 1 staminoid petal (black arrowhead), 8 stamens and a pistil formed by 3 unfused carpels. (E) Lateral view of a type I *ufo-2 35S::UFO*$_{ind}$ flower with the 4 first whorl sepals removed to reveal the inner 2 sepals (arrowheads). (F) Type II *ufo-2 35S::UFO*$_{ind}$ flower with 4 sepals, 2 petals, 1 staminoid petal (arrow), 1 petaloid stamen (arrowhead), 5 stamens (only 2 clearly visible) and a central pistil with 3 partially unfused carpels. (G) Type III *ufo-2 35S::UFO*$_{ind}$ flower with 4 sepals, 4 petals, 6 stamens and a pistil with two carpels. (H) Type IV *ufo-2 35S::UFO*$_{ind}$ flower with 4 first whorl sepals, 1 petal, 6 stamens and a pistil with two carpels. (I) Lateral view of a type IV *ufo-2 35S::UFO*$_{ind}$ flower with 2 of the first whorl sepals removed to reveal the 4 inner stamens and a pistil with 2 carpels. Note the absence of second whorl organs. (J) SEM of a type I *ufo-2 35S::UFO*$_{ind}$ flower. One sepal has been removed to show the inner organs. (K) Detail of J showing 3 stamens in an outer whorl (arrows) and 3 stamens in an inner whorl (arrowheads). (L) Type I *ufo-2 35S::UFO*$_{ind}$ flower with outer organs removed showing the central pistil unfused along two sides. One of the unfused margins has a thickening of the distal part with cell types similar to the anther (compare black and white arrowheads) and cells similar to the filament of the stamen (compare black and white arrows) form the proximal region. (M) Type I *ufo-2 35S::UFO*$_{ind}$ flower with outer organs removed to expose a central pistil formed by three carpels completely unfused on one side. Arrowhead points to an abnormal ovule. (N) Detail of L showing an abnormally shaped ovule. (O) Type IV *ufo-2 35S::UFO*$_{ind}$ flower. No visible petals are present between the sepal whorl and the stamens. (P) Dissected type IV *ufo-2 35S::UFO*$_{ind}$ flower with the sepals removed. No reduced petal is visible. The arrow points to the insertion point of a removed sepal. The arrowheads indicate 2 stamens. A-I are at the same magnification. Bars: 1mm for A-M and O; 100 μm for N and P.

in an average of approximately one flower per inflorescence whereas prolonged induction led to additional restored flowers.

To correlate the duration of induction with the duration of *UFO* expression, we performed quantitative RT-PCR on *ufo-2 35S::UFO*$_{ind}$ apices after an 8-hour vapour induction (Fig. 3A-C). The *ufo-2* allele bears a point mutation that creates a new *Afl*III restriction site (Lee et al., 1997), producing a polymorphism that could be used to determine the ratio of mutant and wild-type forms of the *UFO* transcript (Fig. 3D). Low levels of *UFO* mRNAs were detected before induction (Fig. 3A-C), and both transcripts contributed to this at comparable levels (Fig. 3D). It must be noted, however, that the amount of the transgene transcript should be lower in individual meristem cells than the amount of the endogenous transcript as only a few cells express the endogenous gene whereas the transgene, indirectly driven by 35S promoter, is ubiquitously expressed. As illustrated in Fig. 1, basal transgene expression in the absence of inducer was not sufficient to restore the flowers. High levels of *UFO* transcript, due to the activation of the transgene, were observed from 8 to 24 hours (Fig. 3A-D). The level of the transgene mRNA dropped to non-induced levels after 48h. The limited number of restored flowers following a short pulse of induction suggests that the UFO protein is not stable.

Table 1. Effect of duration of ethanol treatment on phenotypic complementation

Ethanol treatment	Number of flowers with restored petals and stamens
0	0
1 day	0.95±0.24
2 days	1.53±0.29
3 days	2.00±0.25
5 days	2.21±0.28

Plants were induced by daily 8-hour ethanol vapour treatments for the indicated durations. The mean number of flowers with complemented whorls 2 and 3 per apex was counted ±s.e. At least 17 plants analyzed for each condition.

We also used RNA in situ hybridisation to determine the spatial expression pattern of *UFO* before and after induction in *ufo-2 35S::UFO*$_{ind}$ apices and compared it to expression in wild-type and non-transformed *ufo-2* apices (Fig. 4). As described previously (Ingram et al., 1995; Lee et al., 1997), *UFO* expression is first visible in the centre of stage 2 flowers, when the floral meristems are clearly separated from the apical meristem [stages as defined by Smyth et al. (Smyth et al., 1990)] and then in a cup-shaped domain (Fig. 4F). During stage 3, *UFO* is expressed in a ring of cells interior to the emerging sepal primordia and corresponding to the domains of whorls 2 and 3 (not shown). During stage 4, when the sepal primordia start to grow over the floral meristem, *UFO* expression is concentrated in the presumptive sites of petal primordium initiation (Fig. 4A-F). Faint *UFO* expression can be seen in a small group of cells that correspond to the petal primordia (arrowheads in Fig. 4D,E). In non-transformed *ufo-2* and non-induced *ufo-2 35S::UFO*$_{ind}$ apices, *UFO* was initially expressed in a cup-shaped domain at stage 3, as in wild type (Fig. 4H). Later, *UFO* was expressed at the base of primordia located internal to the sepals (Fig. 4I). By contrast, induced *ufo-2 35S::UFO*$_{ind}$ plants showed ubiquitous expression 24 hours after the beginning of an 8-hour ethanol pulse (Fig. 4J,K). In most, but not all, of the observed apices, the endogenous pattern could no longer be recognised, suggesting that the local level of transgene expression was similar to or higher than the expression of the endogenous gene.

Thus, following ethanol induction *UFO* expression is rapidly and uniformly increased in *ufo-2 35S::UFO*$_{ind}$ apices. Furthermore, a single pulse of *UFO* expression lasting 24 to 48 hours is sufficient to restore normal development in whorls 2 and 3.

Phenotype of second and third whorl organs defines different phases of restoration

We reasoned that the partially complemented flowers that developed above and below the restored flowers could reveal different functions of *UFO* that could be temporally separated. In flowers forming below the restored ones, *UFO* expression was induced at a later stage of development than in the complemented ones, whereas in those above, *UFO* was expressed at an earlier stage. Therefore we defined the sequence of floral phenotypes arising following a 5-day ethanol treatment of *ufo-2 35S::UFO*$_{ind}$ inflorescences. Based on the second and third whorl organs phenotype, 4 types could be defined, arising along the stem from bottom to top.

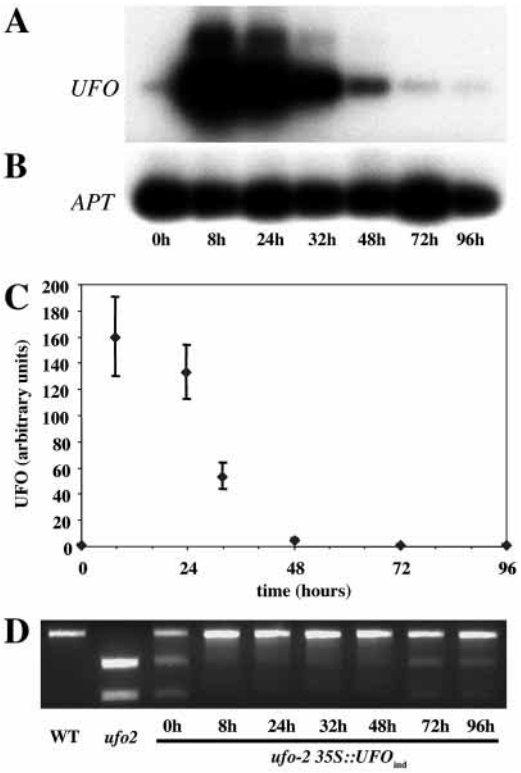

Fig. 3. Kinetics of *UFO* expression following an 8-hour pulse of ethanol induction in *ufo-2 35S::UFO*$_{ind}$ apices. (A,B) The expression levels of *UFO* (A) and the control *APT* (B) were analysed by RT-PCR at the indicated times following the beginning of an 8h-vapour induction in *ufo-2 35S::UFO*$_{ind}$ apices. (C) The expression level of *UFO* was quantified as a ratio of the level of *APT* and was given the arbitrary value 1 at time 0h. Bars represented SE of 6 replicates. (D) Equal amounts of RT-PCR products obtained after amplification of the *UFO* transcripts from apices of wild-type (WT), *ufo-2*, or *ufo-2 35S::UFO*$_{ind}$ plants following an 8-hour ethanol induction were digested by *Afl*III to discriminate between wild-type and mutated transcripts.

In the most basal part of the stem, typical *ufo-2* flowers were formed, with no, or very few, petals and stamens. Restoration of stamen identity and partial restoration of petal identity, was the first sign of partial restoration and defined **type I** flowers (Table 2, Fig. 2C-E,J-N). Chimeric sepal/petal organs were present in type I flowers (Fig. 2C-E) indicating that petal identity had not been fully restored. Petal/stamen organs were observed (Fig. 2C,E) that may have resulted from abnormal alignment of the organ primordia with the underlying expression patterns of the organ identity genes. Although the average number of stamens per flower was close to that observed in the wild type, the number of stamen or petaloid stamens in individual flowers varied from 3 to 12. When more than 7-8 stamens were present they occupied 2 whorls (Fig. 2J,K), reminiscent of the *superman* mutant (Bowman et al., 1992; Schultz et al., 1991). The total number of organs was increased from 14.2 per *ufo-2* flower to 17.8 in type I (Table 2). Therefore, besides modifying the identity of pre-existing primordia, *UFO* expression induced a change in the pattern of primordia initiation and/or growth.

In **type II** flowers, sepal/petal chimeric organs were no

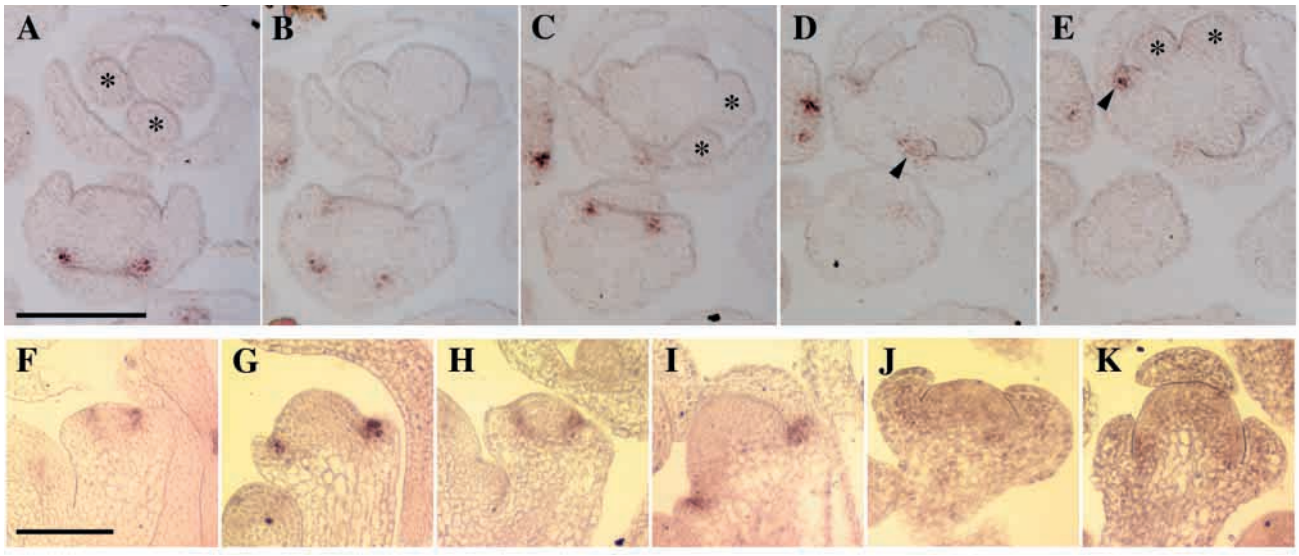

Fig. 4. *UFO* expression pattern during flower development.The expression pattern of *UFO* was examined by RNA in situ hybridisation in wild type (A-G) and in *ufo-2 35S::UFO*$_{ind}$ before ethanol induction (H,I) and 24 hours after the beginning of an 8-hour ethanol pulse (J,K). (A-E) Transverse sections, (F-K) longitudinal sections. (A-E) *UFO* is expressed in four discrete spots corresponding to expected positions of the petals. The expression subsists weakly in the petal primordia after their initiation in a stage 6 flower (arrowheads). *, stamens. (F) In wild-type early stage 3 flowers, *UFO* is expressed in a cup-shaped domain. (G) A stage 4 wild-type flower with *UFO* expressed at the boundary of the meristem and the sepals. (H) *UFO* is expressed in a non-induced *ufo-2 35S::UFO*$_{ind}$ early stage 3 flower in a cup-shaped domain similar to the wild-type. (I) In a non-induced *ufo-2 35S::UFO*$_{ind}$ stage 4/5 flower, *UFO* is expressed at the axils of the sepals. (J,K) 24 hours after an 8-hour ethanol vapour treatment, *UFO* is uniformly expressed in a *ufo-2 35S::UFO*$_{ind}$ early stage 4 flower (J) or in a stage 4/5 flower (L). A-E are at the same magnification and F-K are at the same magnification. Bars: 100 μm.

Table 2. Floral organs in *ufo-2* and different phases of phenotypic complementation

		Floral organs												
	n	1st W-Sep	Sep	Sep/Pet	Pet/Sep	Pet	Pet/St	St/Pet	St	unf Ca	Fil	div. St	4th W Ca	Total
ufo-2	150	3.85	1.95	0.31	0.04	0.02	0.06	0.02	0.35	0.62	3.21	0.70	3.00	14.14
Type I	14	4.00	0.64	1.14	0.86	0.93	0.64	0.50	5.64	0*	0*	0*	3.43	17.79
Type II	22	3.82	0*	0*	0*	1.91	0.82	1.45	5.45	0*	0*	0*	3.18	16.64
Type III	26	4.04	0*	0*	0*	4.00	0*	0*	5.23	0*	0*	0*	2.60	15.87
Type IV	38	3.97	0*	0*	0*	0.21	0.16	0.79	4.18	0*	0*	0*	2.24	11.55

The 30th first flowers were counted in *ufo-2* apices.

The floral organs in flowers at different phenotypic complementation phases after induction were counted.

n, number of flowers analysed.

1st W-Sep, first whorl sepals; Sep, sepals; Sep/Pet, sepals-petals chimeric organs: overall sepaloid organs with some white petaloid streaks; Pet/Sep, petals-sepals chimeric organs: overall petaloid organs with some green sepaloid streaks; Pet, petals; Pet/St, petals-stamens chimeric organs: overall petaloid organs with some yellow anther tissues; St/Pet, stamens-petals chimeric organs: stamen like stalked organs with some white petaloid tissues; unf Ca, unfused or partially fused to the pistil carpeloid organs; Fil, filmentous organ; div St, divers stamen like organs: stamen like organs with some sepaloid, carpeloid or sepaloid and petaloid features; 4th W Ca, 4th whorl carpels.

*The number of this class of organs is fixed by the definition of the phase of phenotypic complementation.

longer formed. In the inner whorls, only petals, stamens and petal/stamen chimeric organs developed, as seen in the weak *ufo-6* mutant (Levin and Meyerowitz, 1995) (Table 2, Fig. 2F). This suggests that in contrast to type I flowers, B activity is fully restored in type II flowers. The presence of petal/stamen chimeric organs indicates that coordination of growth patterns with petal/stamen identity boundary is not fully established in these flowers as in type I flowers. Flowers containing an extra whorl of stamens could be observed at a lower frequency than in type I flowers.

Whorls 2 and 3 were fully restored in **type III** flowers (Table 2, Fig. 2G). Most of type III flowers had 4 petals but occasionally 5 petals formed, whereas the stamen number was more variable ranging from 3 to 8. The increase in the number of petals and stamens has already been reported in *35S::*UFO lines and is related to the overexpression of *UFO* (Lee et al., 1997).

Type IV flowers were characterised by a single whorl of 4-8 stamens or petaloid stamens interior to the sepals (Table 2, Fig. 2H,I,O,P). Petals were absent, with the exception of an occasional petal or staminoid petal in some flowers. No signs of reduced petals were visible at the expected empty position interior to the sepals (Fig. 2I,O,P). Accordingly, the total number of floral organs was reduced to 11.6 in type IV flowers whereas it was 15.9 in type III.

Approximately 75% of the non-*ufo*-like flowers induced by

Table 3. Pistil and ovules in *ufo-2* and different phases of phenotypic complementation

	Pistil			Ovules		
	n	2 carpels*	Unfused†	*n*	Abnormal‡	Abnormal on unfused pistil§
ufo-2	150	25%	9%	0	13%	0%
Type I	72	24%	69%	58	34%	33%
Type II	52	46%	31%	30	20%	17%
Type III	61	67%	16%	35	9%	6%
Type IV	38	85%	3%	28	0%	0%

*Percentage of flowers with pistil formed by two carpels.
†Percentage of flowers showing fusion defects between carpels.
‡Percentage of flowers with at least one abnormal ovule.
§Percentage of flowers with at least one abnormal ovule developing and a pistil with fusion defects.

Table 4. Developmental stages of the five oldest floral buds after long days (LD) floral inductive treatment

	Floral stage				
LD duration	S6	S4-5	S3	S2	S1
12	5.0	0	0	0	0
11	5.0	0	0	0	0
10	3.7	1.3	0	0	0
9	0.7	3.5	0.5	0.3	0
8	0	0.1	2.3	2.6	0
7	0	0	0.1	4.7	0.2*
5	0	0	0	0	5.0*

Plants kept under short days conditions were induced for flowering for various durations by long day conditions (LD duration, in days). The number of floral buds at the given developmental stage amongst the five oldest flowers not subtended by a leaf was recorded in 10 plants.
S2, S3, S4-5, S6+, average number of floral buds at stage 2, 3, 4 or 5, and 6 or more per apex. Floral stages defined by Smyth et al. (1990).
*Deduced as the total number of floral meristems minus total of S6+ to S2 stages.

ethanol fitted clearly into this classification of types I to IV. The 25% remaining flowers were either transitional forms between the different types, or flowers showing weaker restoration, where one or more 'unusual organs' such as filaments or unfused carpels remained.

The order of the types was conserved in all the inflorescences observed but the number of flowers in each type varied from plant to plant and sometimes one or more types could be missing. The average number was 1 type I flower, 1-2 type II flowers and 2-3 type III and IV flowers.

Phenotype of whorls 1 and 4 during the different phases of restoration

Having established a logical and reproducible framework for phenotypic analysis based on the morphology of whorls 2 and 3, we then analysed the effect on whorls 1 and 4. There was no significant difference in the first whorl between the different flower types, except that petaloid sepals could be observed occasionally in the outer whorl of type IV flowers (results not shown).

The number of carpels forming the pistil decreased progressively from type I to type IV flowers (Table 2). Similarly, the percentage of flowers with a wild-type two-carpel pistil increased from 24% in type I flowers to 85% in type IV (Table 3). Whereas only a small minority of pistils showed fusion defects in *ufo-2* flowers, 69% of type I flowers showed such defects, which often extended along the whole pistil (Fig. 2C,D,M). Fusion defects usually only affected one side of the pistil, but occasionally two defects on opposite sides of the pistil could give rise to two groups of carpels united only at the style (Fig. 2L). Thickening at the margins of the non-united carpel walls could be observed, mainly in the distal half of the pistil. A structure resembling an anther could form in the distal part of the unfused margin whereas elongated cells resembling stamen filament cells formed on the proximal part of the pistil margin (Fig. 2L). This suggested that the margins had partial stamen identity. Fusion defects became less frequent in later arising types and were rare in type IV flowers.

Occasionally, abnormal ovules could be observed in type I to III flowers (Table 3, Fig. 2M,N). These ovules had a straight elongated tubular shape as compared to wild-type ovules which have a typical S shape. The number of abnormal ovules was variable, ranging from one to six, and most often was one to two per pistil. Abnormal ovules could be observed in about one third of the type I flowers and became rarer in later types. Most of the abnormal ovules developed in pistils with fusion defects (Table 3). There was no obvious correlation of the position of these ovules with the proximodistal axis of the pistil or the unfused pistil margins.

In conclusion, specific effects on the fourth whorl and ovules are observed during the different phases of phenotypic restoration, suggesting that *UFO* proper expression is critical for the development of these structures.

Different phases of restoration reflect induction of *UFO* at specific stages of floral development

The different phases of restoration could be the consequence of fluctuations in the levels of *UFO* expression. Full restoration in type III flowers could follow high expression of *UFO*. The early and late types could result from weaker expression levels during the time course of induction and arrest of *UFO* expression. Alternatively, the different phenotypes could result from induction at different stages of floral development. In order to distinguish these possibilities, we induced *UFO* expression at different developmental stages. According to the first hypothesis, a similar range of phenotypes would be expected. In the second case, one would expect a correlation between the stage at the time of induction and the phenotype of the mature flower.

ufo-2 35S::UFO$_{ind}$ were kept in a vegetative state by growing them under short day conditions and then they were induced to flower by transferring them to long days (LD). After different durations in LD, plants were treated with 1% ethanol for 5 days. The stage of the five oldest floral buds not subtended by a leaf was determined at the moment of induction using a binocular microscope or scanning electron microscope (Table 4, Fig. 5) and related to the final morphology of the flowers. We excluded floral meristems subtended by a leaf from our analyses as they appeared sometimes less developed than more apical meristems. A simplified classification of the floral buds stages was used: stages 2 and stage 3 were as defined by Smyth et al. (Smyth et al., 1990), stage 4-5 corresponds to stages 4 and 5 from Smyth et al., and stages 6+ had the floral buds enclosed by the sepals (stages 6 or more from Smyth et al.).

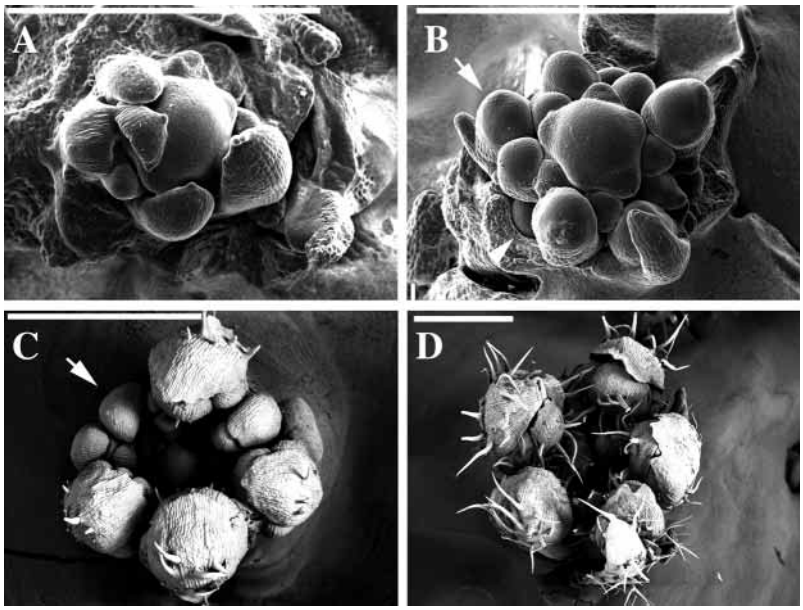

Fig. 5. Floral induction of *ufo-2 35S::UFO*$_{ind}$ by LD treatment. *ufo-2 35S::UFO*$_{ind}$ plants were kept under short day conditions and induced for flowering by long day conditions. (A) Apex after 5 LD. No recognisable floral meristem is visible. (B) Apex after 8 LD. Arrowhead points to a floral meristem at stage 2 subtended by a leaf. Arrow indicates an early stage 3 flower in the axil of a leaf-like structure. The oldest floral meristems are at stage 3. (C) Apex after 10 LD and (D) apex after 12 LD. The oldest floral meristems have reached stage 6 or more. Arrow in C points to a flower with reduced growth. Bars: 500 μm.

No recognisable floral meristems were visible after 5 LD (Fig. 5A), but extrapolation of the total number of floral buds formed, assuming a constant primordia initiation rate, indicated that the first stage 2 meristem was formed between 5 and 6 LD. After 7 LD, mainly stage 2 flowers were visible. After 8 LD approximately half of the five oldest flowers were at stage 3 and half at stage 2 (Table 4, Fig. 5B). After 9 LD, the majority of the five oldest flowers were at stage 4-5. Most of the flowers had reached stage 6+ after 10 LD and all had after 11 LD or 12 LD. (Table 4, Fig. 5C,D).

ufo-2 35S::UFO$_{ind}$ flowers induced during stage 6+ (12 LD) developed with a general *ufo-2* phenotype, showing only a slight increase in the number of stamens and more petaloid characteristics in the sepaloid organs (Fig. 6A). Therefore, expression of *UFO* had little effect at or after stage 6+.

Type I flowers were occasionally observed in 11 LD apices and more often in 10 LD and 9 LD apices (Table 5, Fig. 6B). This suggests that stamen identity could be restored in early stage 6+ and stage 4/5 but that at this stage, the identity of the second whorl organs was already fixed and could not be altered by *UFO* expression. It must be noted that the endogenous *UFO* gene is not expressed at these stages of development in the stamen whorl and that the prolonged competence of the stamens to respond to UFO is revealed here by the ectopic expression resulting from the use of 35S promoter.

A significant number of type II flowers were observed in inflorescences of 9 LD plants (Table 5) and developed from floral buds induced at stage 3. Restoration of the petal and stamen identities in type II flowers suggests that B genes were fully functional and that the identities of the corresponding meristem domains giving rise to them had not been fixed at the time of induction. The presence of chimeric petal/stamen organs suggests, however, that the developing floral organs primordia in these whorls are misaligned with the underlying organ identities genes, especially those of class A and C. Defects in gynoecium development occur most often in type I and II flowers (Table 3). Type I and II flowers result from *UFO* expression at or after stage 3, when endogenous UFO is not detected in the central domain of the flower giving raise to the fourth whorl. Therefore the gynoecium defects are likely to result from the ectopic expression of *UFO* in the central domain due to the use of the ubiquitous 35S promoter.

Table 5. Effect of *UFO* induction at different stages of floral development

	Phases of phenotypic complementation					
LD duration	Mutant	Type I	Type II	Type III	Type IV	ND
12	4.9	0.1	0	0	0	0
11	4.5	0.4	0	0.1	0	0
10	3.1	1.1	0.1	0.2	0	0.5
9	1.5	1.7	0.6	0.3	0	0.9
8	0.2	0.5	0.9	1.9	1	0.5
7	2.3	0.1	0	0	1.8	0.9
5	4.5	0	0	0	0.1	0.4
No ethanol	5	0	0	0	0	0

Plants kept under short days conditions were induced for flowering for various durations by long day conditions (LD duration, in days) before ethanol induction.

No ethanol, control plants which have not been induced by ethanol.

The five oldest flowers not subtended by a leaf were ordered into the complementation types. ND, not determined. 15 plants were analyzed for each condition.

A significant number of type III flowers were observed after induction of 8-LD plants and they resulted from the expression of UFO in stage 2 meristems (Table 5, Fig. 6C). Therefore, expression of *UFO* as early as stage 2 is required for proper development of whorl 2 and 3 organs.

Type IV flowers were observed in apices induced after 7 or 8 LD (Table 5, Fig. 6C,D). They resulted from the expression of *UFO* in early stage 2 and stage 1 floral primordia. Such flowers were not observed following *UFO* expression in a wild-type background. Therefore, they did not simply result from ectopic *UFO* expression too early, but were the consequence of a short exposure to *UFO* and a lack of *UFO* expression during later stages. The absence of visible petals in type IV flowers suggests that a late function of *UFO* is to

promote growth of the petals. Type IV flowers also showed the best restoration of pistil development, demonstrating that *UFO* expression as early as stage 2 is required for this process.

Induction of *UFO* at 5 LD had no major effect on floral morphology compared to non induced flowers (Table 5, Fig. 6E).

Sequential functions for *UFO*

We show that *UFO* has a number of different and spatially distinct roles during flower development (Fig. 7). In wild type, the first function of *UFO* is during stage 2, when it is expressed in the centre of the flower and is required for the patterning of the flower. At this stage, *UFO* regulates the position of the primordia of whorls 2, 3 and 4. The second function is to establish the identity of the petal and the stamens and this may occur at the same stage, but can still be determined during stage 3, when *UFO* is expressed in presumptive whorls 2 and 3. A third and previously unsuspected function occurs after petal identity is specified, where *UFO* is required for initial petal outgrowth. Finally, *UFO* appears to regulate the size of the third whorl.

DISCUSSION

Conditional expression, a new tool to dissect complex developmental processes in plants

In this paper, we have used a chemically inducible expression system to conditionally restore a developmental mutant, *ufo*, thereby dissecting its complex phenotype and revealing new functions. Mutants are powerful tools to study and define gene function, but simple loss-of-function mutants can have severe limitations when a given gene has sequential or interdependent functions that may be epistatic to one another. Conditional mutants largely overcome such problems but such alleles are not available for most genes. Ethanol-inducible expression was used here to ubiquitously supply *UFO* gene product at different but defined developmental stages of flower development and thus temporally dissect the functions of *UFO* during early flower development. Temporal dissection of *UFO* function is particularly appropriate because *UFO* is expressed in a very dynamic pattern, and has been proposed to regulate multiple aspects of floral development including control of organ identity and primordia initiation patterns. These two previously identified roles of *UFO* have been separated here and assigned to specific stages of flower development. Furthermore, transient *UFO* expression leads to flowers missing second whorl organs, a phenotype not observed in the loss-of-function or constitutive overexpressors lines, revealing an additional role of *UFO* in promoting initial outgrowth of the petals. Because we used the ubiquitous 35S promoter, we could also analyse the effect of ectopic UFO expression. This allowed us to study the effect of delayed activation of *UFO* in domains where it should be expressed, for instance in petals and stamens and in domains where UFO is only very transiently expressed such as the centre of the flower. Thus, the competence of tissues to react to *UFO* at different developmental stages could be tested.

UFO promotes B activity but the response windows differs between petals and stamens

Induction of *UFO* expression from stage 2 or stage 3 onwards restores full B activity as shown by restoration of petal and stamen identities (Fig. 7A3,A4). As the cells giving rise to petals and stamens express the endogenous *UFO* gene during stages 2 and 3, this is consistent with a role for *UFO* in defining the identity of the B whorls during this period. UFO is part of a larger protein complex that promotes B activity, probably by targeting a negative regulator of B expression for specific protein degradation (Samach et al., 1999; Zhao et al., 1999; Zhao et al., 2001). Thus, absence of UFO may result in excess negative regulator and therefore, failure to accumulate B-function.

However, our timed induction experiments indicate that the definition of petal identity has the more stringent requirements

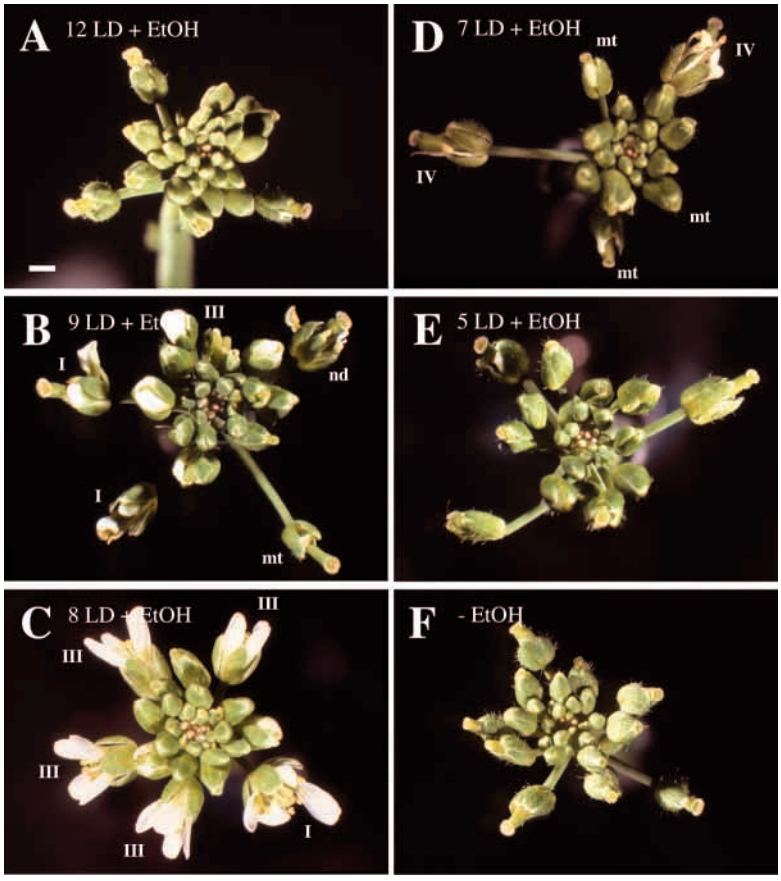

Fig. 6. Induction of *UFO* expression at different floral stages of *ufo-2 35S::UFO*$_{ind}$. *ufo-2 35S::UFO*$_{ind}$ plants were ethanol-induced (A-E) after various durations of floral induction by long day treatment or were not ethanol-induced (F). Ethanol induction after 12 LD has no major effect on floral morphology of the five oldest flowers (A). Induction after 9 LD leads to a majority of type I flowers (B). After 8 LD, ethanol induction leads to a majority of type III flowers (C). Type IV and mutant-type (mt) flowers are observed following induction after 7 LD. (D). Mutant-type flowers develop on treated apices after 5 LD (E) as in non-induced plants (F). nd, flower that could not be classified as a type I-IV or a mutant flower. Bar: 100 μm.

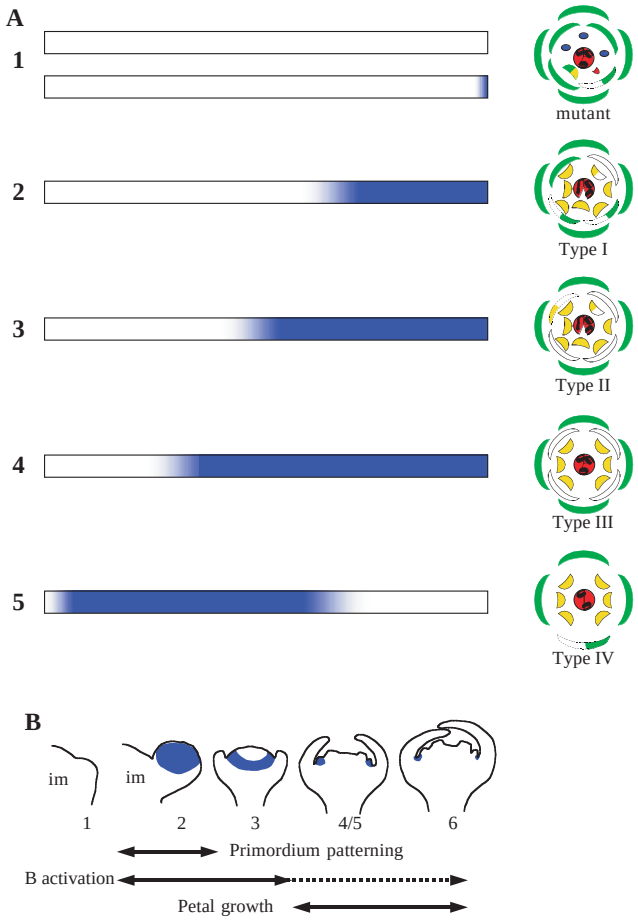

Fig. 7. Summary of floral phenotypes and model of the role of *UFO* during flower development. (A) Effect on the flower morphology of the timing of *UFO* induction (blue shading in bars represents the window of *UFO* expression) in the *ufo-2 35S::UFO*$_{ind}$ line. In the floral diagrams, green represents sepals, white petals, yellow stamens, red carpels, blue filaments and black ovules. Bi-color represent chimeric organs. (1) In the absence of *UFO* induction or expression later than late stage 6, flowers with a mutant phenotype are produced. (2) Ubiquitous induction of *UFO* during the floral stage 4/5 or early 6 leads to stamen identity restoration and partial restoration of petal identity. An extra whorl of stamens, carpel fusion defects and abnormal ovules are observed in some flowers. (3) Ubiquitous *UFO* expression from the floral stage 3 onwards leads to full restoration of petal and stamen identities. The organ primordia and the floral patterning are however misaligned. An extra whorl of stamens, carpel fusion defects and abnormal ovules are observed in some flowers. (4) Ubiquitous *UFO* expression starting at floral stage 2 leads to complete restoration of petal and stamen development. Pistils show occasional increased carpel number or fusion defect. (5) Transient ubiquitous *UFO* expression from early floral stage 2 onwards or earlier leads to normal stamen development but absence of petal growth. Pistils are normal. Petaloid sepals develop occasionally. (B) Diagram of the early stages of flower development with the domain of *UFO* expression shown in blue. The timing of the different role of *UFO* is represented below. Proper patterning of the primordia in the three inner whorls requires *UFO* expression during the floral stage 2. The B function activity requires *UFO* expression during stage 2 and/or 3 but can be induced as late as stage 6 by ectopic *UFO* expression (dotted line). Proper petal growth requires later *UFO* expression.

for *UFO*. If *UFO* is supplied late in development, starting at stage 4/5 or at early stage 6, petal identity is only partially restored, whereas stamen development is fully restored across a wide time-span (Fig. 7A2) Therefore, petal primordia lose their competence to respond to *UFO* expression before the stamen primordia. This could result from a failure of *UFO*-mediated activation of the B genes in petal primordia. Alternatively, the B genes could be activated equally in both petal and stamen primordia, but petal primordia may have a more stringent temporal or quantitative requirement for B activity. There is some evidence that defining petal identity does have a higher requirement for B function in that petals of the temperature sensitive *ap3-1* mutant grown at 16°C, where AP3 is partially functional, have sepaloid characteristics whereas stamens develop normally (Bowman et al., 1989). Thus, delayed activation of *UFO* expression during stage 4 to early 6 may lead to low levels of B activity sufficient for stamen but not for petal identities.

Early *UFO* expression during stage 2 is required for proper primordia patterning in whorls 2, 3 and 4

Although induction of *UFO* during stages 2 or 3 is equally able to restore B activity, *UFO* expression during stage 2 is required for correct spatial coordination between primordia initiation patterns and the underlying identity gene expression patterns (Fig. 7A4). The endogenous *UFO* gene is expressed in a continuous domain covering the presumptive whorls 2, 3 and 4 during stage 2, consistent with such a role.

UFO expression during early stage 2 is also required for normal patterning of the fourth whorl organs. Pistils of *ufo* flowers show an increased number of carpels, which can be best corrected by *UFO* induction during stage 2, when endogenous *UFO* expression includes the presumptive fourth whorl cells. Lack of whorl 4 defects as a result of *UFO* induction persists even up to stage 6, but with a progressively decreasing efficiency. This long window of competence is in sharp contrast to the narrow window during stage 2 for proper initiation patterns of petals and stamens. This could be explained by a *UFO* requirement before the formation of the primordia as the petal and stamen primordia are initiated before the carpel primordia. Alternatively *UFO* could directly regulate primordia patterning in whorl 2 and 3 and have an indirect role in whorl 4 by diminishing the pool of meristematic cells available for primordium recruitment. In such an hypothesis, the effect of *UFO* on primordium patterning in the fourth whorl would be comparable to those of genes such as *CLAVATA* that regulate the size of the meristem (Clark et al., 1993; Clark et al., 1995).

UFO promotes petal outgrowth, a novel role revealed by transient expression

Transient *UFO* expression during the very early stages of flower development leads to an unexpected phenotype, where mature petals are absent (Fig. 7A5). These flowers are not simply the consequence of an early ectopic expression as they are not observed in either constitutive 35S::*UFO* lines (Lee et al., 1997) or after ethanol induction of *UFO* in a wild-type background. Absence of second whorl organs is never observed in *ufo* mutants, where sepal-like organs replace the petals (Levin and Meyerowitz, 1995; Wilkinson and Haughn,

1995). Therefore, this novel floral phenotype results from the transient expression of *UFO* during early stages of floral development demonstrating that prolonged *UFO* expression is required for petal growth.

The lack of petals in the mature flower could be due to an absence of the second whorl resulting from defects in the early patterning of the flower into whorls. Although this cannot be ruled out it seems unlikely as patterning into whorls occurs during stage 3, whereas the absence of petals results from deficiency of *UFO* expression after stage 3. Alternatively, the absence of petals may result from defects in primordia initiation or outgrowth. Occasionally a single wild-type petal is present following transient *UFO* expression. Smaller or misshapen petals are never produced, suggesting that UFO has a triggering effect on primordia initiation or early outgrowth and that later growth becomes independent of the presence of UFO. During wild-type development, endogenous *UFO* gene expression occurs in the petal primordia and persists at their abaxial base throughout this period consistent with our conclusion that *UFO* is directly required for petal growth.

What might be the mechanism by which *UFO* promotes petal growth? *UFO* is required specifically for the growth of petals and not for other organs arising in the second whorl because *ufo* mutants develop normal sepals instead of petals. *UFO* requirement for petal growth is by-passed when *AP3* and *PI*, the two B function genes, are overexpressed in a *ufo-2* background (Krizek and Meyerowitz, 1996). This suggests that *UFO* promotes petal growth through its positive effect on B activity. Regulation of B expression has been subdivided in two phases, an early establishment phase when the two B genes, *AP3* and *PI*, are activated independently and a later maintenance phase where the two genes maintain themselves (Goto and Meyerowitz, 1994; Jack et al., 1992; Jack et al., 1994). In *ufo* flowers, normal *AP3* and *PI* initial expression patterns have been observed during stage 3, but the expression of *AP3* is reduced compared to wild-type as early as stage 4 (Samach et al., 1999). This defect is earlier than the onset of the autoregulatory circuit. Therefore *UFO* expression could be required to retain high levels of *AP3* expression during the transition from the early establishment phase to the maintenance phase. This involvement of *UFO* may be particularly critical for promoting petal growth.

UFO has previously been proposed to promote growth based on the observation that flowers can be replaced by filaments in *ufo* (Levin et al., 1998; Levin and Meyerowitz, 1995). However, a negative effect of *UFO* on growth was also proposed based on the increased growth of inflorescence or floral meristems or in second whorl organs of *ufo* (Levin and Meyerowitz, 1995; Samach et al., 1999). Therefore the relationship between *UFO* and growth is complex and may be dependent on other developmentally regulated factors.

UFO function requires proper timing of expression

The above observations indicate that proper timing of expression is required for *UFO* to fulfil all of its functions during flower development. Additional evidence for this comes from the floral phenotypes arising from late induction. When *UFO* is activated during stage 4/5 and in a minor way during stage 3, an additional whorl of stamens, carpel fusion defects and abnormal ovule development are observed in some flowers (Fig. 7A3,A2).

Additional stamen whorls and staminoid identity of the unfused carpel margins following activation of *UFO* during stage 4-6 suggest that the B function has spread towards the centre of the meristem and/or there has been an increased proliferation of the third whorl. These defects are reminiscent of the *superman* (*sup*) mutant phenotype (Bowman et al., 1992; Schultz et al., 1991). *SUP* and the B genes seem to antagonistically regulate the size of the stamen whorl (Sakai et al., 1995). Increased stamen whorls observed following late *UFO* activation, therefore, could be due to increased B activity unbalanced by *SUP* activity. The balance between *SUP* and B genes is apparently re-established when *UFO* is induced before stage 3 because additional whorls of stamens are not observed when we induce during early stages of development. The B genes have been shown to positively regulate *SUP* expression (Sakai et al., 1995; Sakai et al., 2000), so early *UFO* expression may allow a proper balance between SUP and B function, leading to normal flower morphology. However, later ectopic *UFO* expression, in the central domains where endogenous *UFO* is no longer detected, is still able to restore B function but the B genes may not be able to restore proper *SUP* function. This would lead to a distorted regulation between SUP and the B genes resulting in an excess of B activity relative to SUP.

Induction of *UFO* expression at different stages of development allows different functions of *UFO* to be separated and assigned to specific developmental windows that generally correlate with the endogenous expression pattern. The effects of *UFO* expression are complex, involving both direct and indirect events. For instance, early *UFO* expression activates the B function and, indirectly, the regulatory feedback loop involving *SUP* and B function. Delayed activation of *UFO* is still able to induce expression of the B function but not the regulatory loop. Therefore controlled *UFO* expression not only allows the temporal requirements of gene expression to be dissected but can also reveal more indirect effects.

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