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The Petunia MADS Box Gene *FBP11* Determines Ovule Identity

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In contrast to the wealth of information relating to genes regulating floral meristem and floral organ identity, only limited data are available concerning genes that are involved in determining and regulating the identity and development of an ovule. We have recently isolated the *floral binding protein 11* (*FBP11*) MADS box gene from petunia and found that it is expressed exclusively in ovule primordia and subsequently in the ovules, suggesting a role for this gene in ovule formation. To test this hypothesis, we constructed a recombinant gene in which the full-size *FBP11* cDNA was placed under the control of a strong cauliflower mosaic virus 35S promoter. Transgenic petunia plants expressing this chimeric gene have ovulelike structures on the adaxial side of the sepals and the abaxial side of the petals. Detailed morphological studies showed that these ovulelike structures are true ovules. RNA gel blot analysis was performed to investigate ectopic *FBP11* expression in relation to the expression of the closely related *FBP7* gene and the putative petunia class C-type homeotic genes *FBP6* and *pMADS3*. Our results indicate that *FBP11* represents an ovule identity gene. A new model describing the mode of action of *FBP11* as an additional class D MADS box gene is presented.

INTRODUCTION

A typical angiosperm flower consists of four different organs, which are organized in whorls: the outer sterile organs (first and second whorls) are the sepals and petals; the inner organs are the stamens and carpels (third and fourth whorls). The carpels contain the ovules, which produce the female gametophyte and are the precursors of the seed.

During the past few years, much has been learned about the molecular processes controlling flower development. From analyses of flower mutants in Arabidopsis (Bowman et al., 1989) and Antirrhinum (Coen et al., 1990; Schwarz-Sommer et al., 1990), a model has been proposed to explain the genetic control of flower morphogenesis. According to this model (for reviews, see Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994), three classes of genes, A, B, and C, define floral organ identity. Class A is required for sepal formation. The combination of A and B results in the formation of petals. B and C together define stamen identity, and C gene function alone leads to carpel formation. Furthermore, A and C genes are postulated to be mutually antagonistic. Although the ABC model can be used to describe the molecular control of flower development, it does not include genes that are specifically responsible for the determination of ovule identity. Limited information is available concerning genes regulating the determination and development of ovules. In two tobacco mutants, one of the pleiotropic effects observed was the homeotic conversion of ovules into stigma–style structures (Evans and Malmberg, 1989). A similar homeotic transformation has also been described for Arabidopsis. In the *bell* mutant, the outer ovule integument is converted into a carpel-like structure (Robinson-Beers et al., 1992; Modrusan et al., 1994; Ray et al., 1994).

We have recently cloned two genes from petunia and have designated them *FBP7* and *FBP11* (for floral binding protein). These genes belong to the MADS box family of transcription factors, and their putative translation products share 90% identity. *FBP7* and *FBP11* are expressed in the center of the gynoecium before ovule primordia are visible. At a later stage, they are exclusively expressed in ovules. Simultaneous inhibition of these two genes, using a *FBP11* cosuppression construct, results in a homeotic transformation of ovules into carpelloid structures, suggesting a role for these genes in the development of ovules (Angenent et al., 1995). However, the mode of action of *FBP11* in ovule determination and the interaction between *FBP7* and *FBP11* remain to be determined.

We investigated the role of *FBP11* in ovule formation by analysis of ectopic *FBP11* expression in transgenic petunia plants. For this purpose, a chimeric gene was constructed in which the complete *FBP11* cDNA was placed under the control of a strong cauliflower mosaic virus (CaMV) 35S promoter.

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Transgenic plants were grown and analyzed morphologically by light microscopy and scanning electron microscopy. Molecular analysis of these plants was performed by RNA gel blot analysis. The interactions between *FBP11* and *FBP7* and between *FBP11* and C-type MADS box genes isolated from petunia were studied. The results presented in this study add to our understanding regarding the mode of action of these genes in determining ovule identity. We propose to expand the ABC model to an ABCD model.

RESULTS

Ectopic Expression of *FBP11* in Transgenic Petunia Plants Resulted in Altered Flower Morphology

To study the mode of action of the *FBP11* gene in transgenic plants, a chimeric gene was made using the *FBP11* cDNA coding region fused to the CaMV 35S promoter containing a tandem duplication of 250 bp, thereby creating a very strong enhancer for plant genes (Kay et al., 1987). Figure 1A shows the T-DNA region containing the chimeric gene construct that was introduced into petunia line W115 by Agrobacterium-mediated transformation. Thirty independent transgenic plants, designated T46001 to T46030, were grown and analyzed. Nine of these plants had abnormal flowers with changes in the morphology of the two outer whorls. RNA gel blot analysis was performed to analyze *FBP11* expression in four transgenic plants: two with wild-type flower morphology and two with aberrant flower morphology. As shown in Figure 1B, two plants (T46008 and T46013) expressed *FBP11* strongly in both leaves and petals, whereas in two others (T46006 and T46022), no ectopic *FBP11* expression was detected. This expression pattern correlated with the phenotype of these plants: T46008 and T46013 showed severe alterations in flower morphology, whereas T46006 and T46022 had the wild-type phenotype.

To analyze the segregation of the transgenes in the progeny, T46008 was backcrossed with wild-type plants. Analysis of 30 offspring showed that the overexpression trait segregated in a Mendelian fashion: 13 progeny plants had the wild-type phenotype and 17 had an aberrant floral morphology. DNA gel blot analysis showed that the transgenes were present in plants with the altered phenotype (data not shown).

Flower Phenotype of the *FBP11*-Overexpressing Plants

Among the nine primary transgenic plants displaying the aberrant phenotype, it was possible to distinguish a more severe phenotype (seven plants) and a mild phenotype (two plants), as shown in Figures 2C and 2B, respectively. The seven transgenic plants with a severely aberrant phenotype for whorls 1 and 2 were analyzed in detail. T46008 is presented as a representative of this class of transgenic plants. In contrast to the wild type, the inner surface of the sepals of T46008 lacks

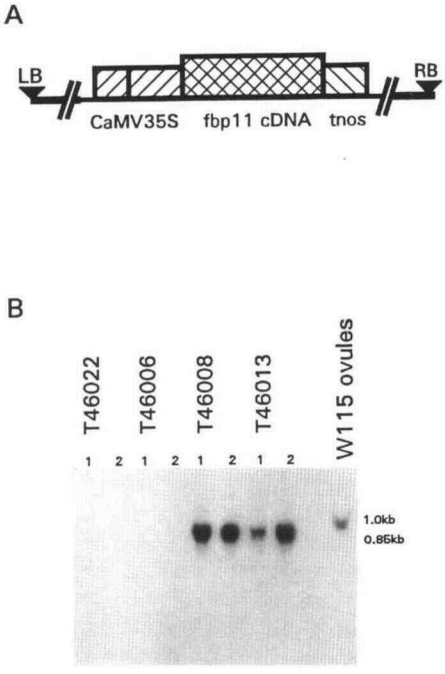

Figure 1. CaMV 35S–*FBP11* Construct and Its Expression in Transgenic Plants.

(A) Chimeric CaMV 35S–*FBP11* gene construct used for transformation. CaMV35S, modified CaMV 35S promoter; LB, left border sequence; RB, right border sequence; tnos, nopaline synthase terminator.

(B) Expression of *FBP11* in four CaMV 35S–*FBP11* transgenic plants. T46022 and T46006 are transgenic plants of normal phenotype, whereas T46008 and T46013 are transgenic plants with very aberrant flowers. W115 is the wild-type petunia line used for the transformation experiment. Total RNA from leaves (lanes 1) and petals (lanes 2) were isolated and hybridized with the ^{32}P-labeled *FBP11* cDNA. The length of the *FBP11* RNA in wild-type ovules (W115) is longer (~1.0 kb) than the chimeric *FBP11* mRNA (0.86 kb) because it contains nontranslated 5′ and 3′ regions that are absent in the CaMV 35S–*FBP11* gene construct present in the transgenic plants.

trichomes, and ovulelike structures are present, as shown in Figures 2E and 2F. The ovulelike structures are always located in the area in which the sepals are fused to each other, possibly due to the high activity of the CaMV 35S promoter in this region. Alternatively, their presence could be explained by the presence in this location of some other required gene product that is not located elsewhere in the sepals or in other parts of the plant. The corolla morphology was also changed. In the early stage of flower development, the petals were unable to cover the stamens and carpels completely (Figure 2D). At the mature stage, the flowers consist only of a tube. In some of the transgenic plants, a few ovulelike structures were found on the outer side of the tube. The two inner whorls and all the vegetative tissues of T46008 were normal.

Figure 2B shows a flower of transgenic plant T46014, a representative of those plants with mild phenotypic alterations of their flowers. The sizes of the limbs of the petals in T46014

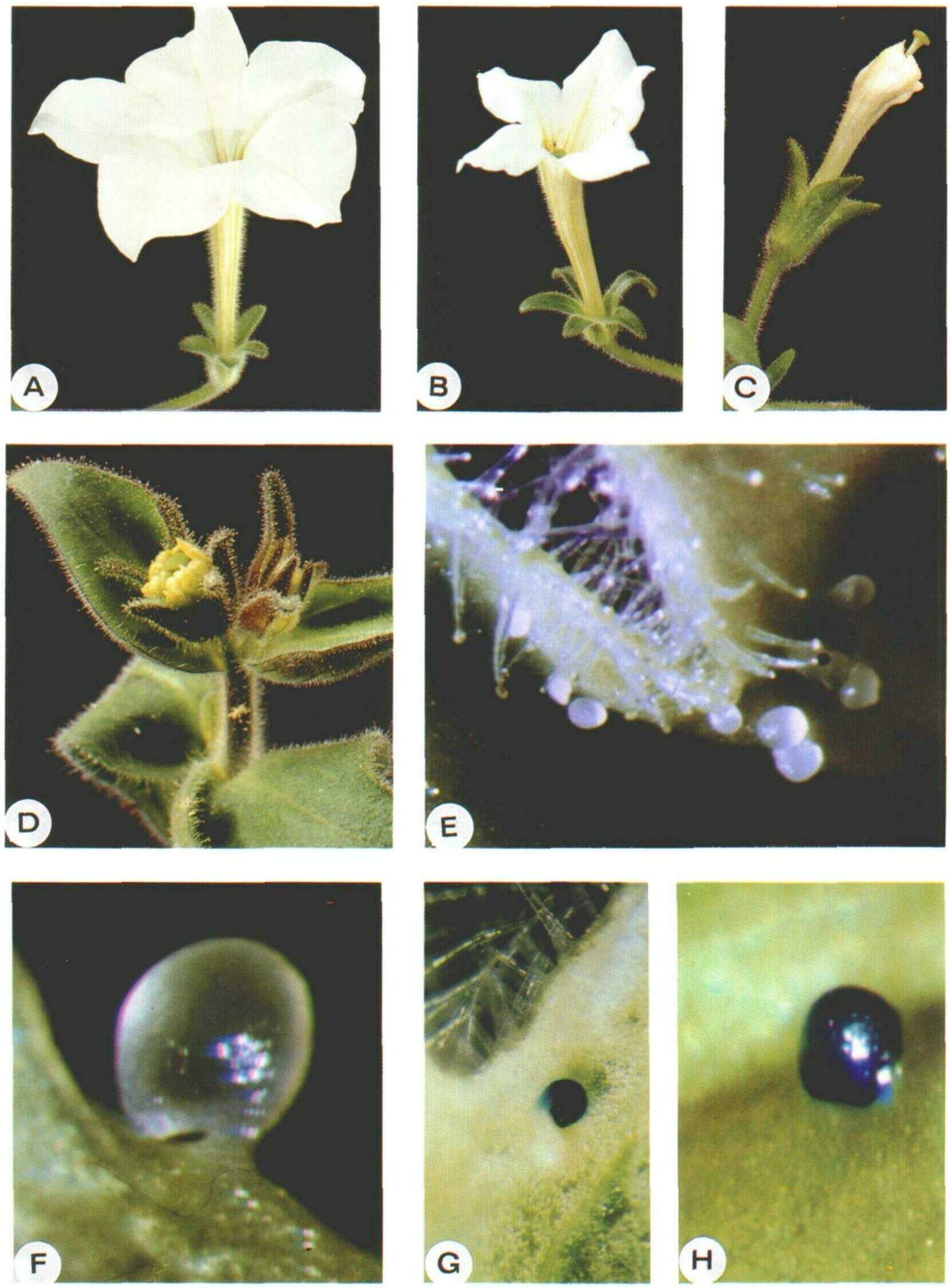

Figure 2. Flower Morphology of Transgenic Plants Ectopically Expressing *FBP11*.

(A) Wild-type petunia flower (W115).
(B) Transgenic plant T46014 showing a mild aberrant phenotype.
(C) Transgenic plant T46008 showing a severe aberrant phenotype.
(D) A young flower bud of T46008.
(E) Sepal adaxial side of T46008. There are only a few trichomes present, and ovulelike structures developed in the area where the sepals are fused to each other.
(F) High magnification of one ovulelike structure.
(G) and **(H)** GUS activity driven by the *chs* promoter in the T46008 genetic background: the *chs*-driven GUS activity in ovulelike structures on the sepals is similar to *chs*-GUS activity in wild-type ovules.

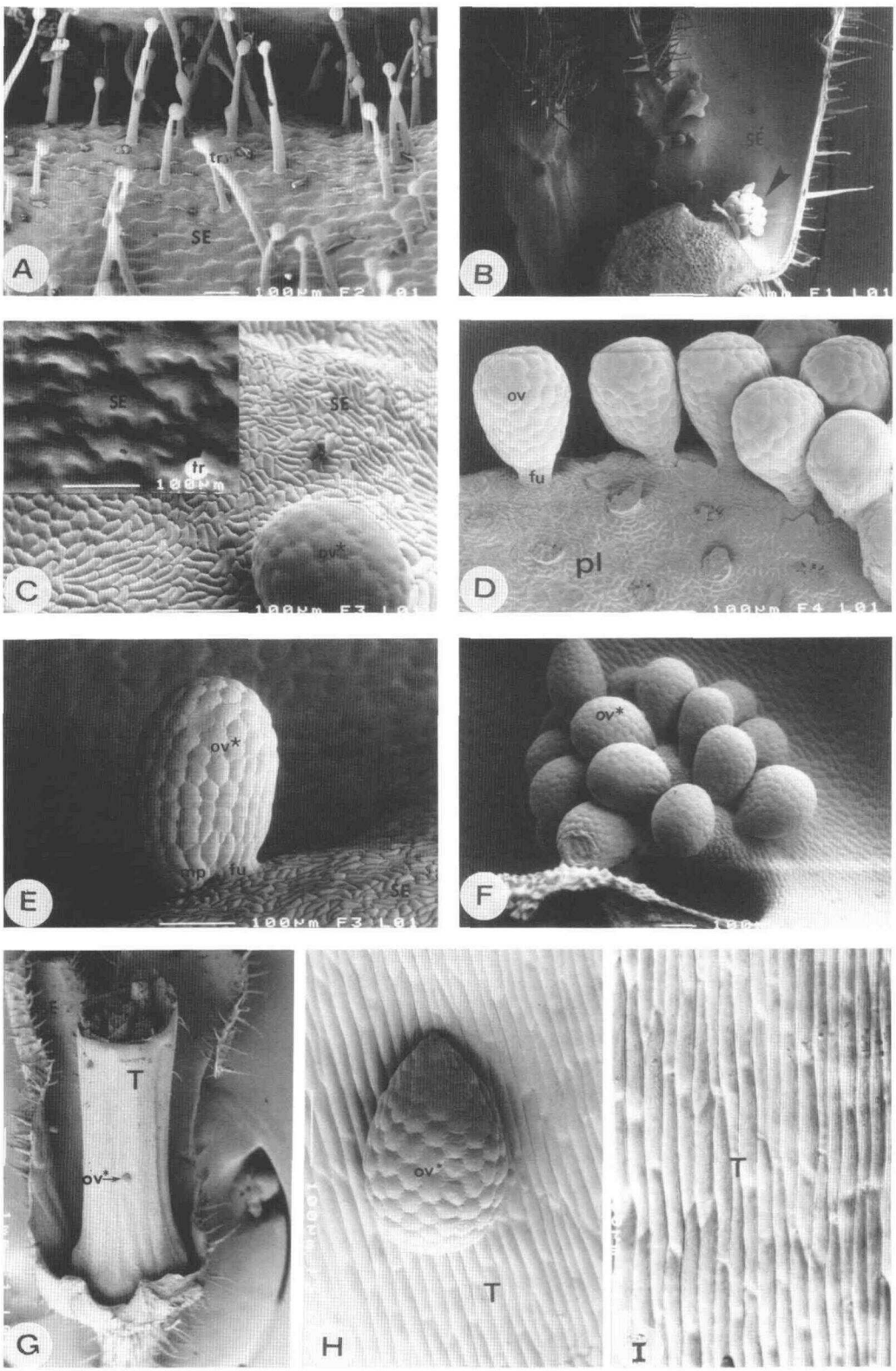

Figure 3. Scanning Electron Microscopy of W115 (Wild Type) and T46008.

are reduced compared with those of W115. The adaxial side of the sepals lacks trichomes, as was observed in T46008; however, no ovulelike structures developed on the sepals of T46014. Besides whorls 1 and 2, all other tissues and organs were normal. This class of transgenic plants was not used for further investigations.

Analysis of T46008 Ovules Using the β-Glucuronidase Reporter Gene Driven by a Chalcone Synthase Promoter

The ovulelike structures that developed on the sepals of the transgenic plants were investigated using the β-glucuronidase (*GUS*) reporter gene driven by a chalcone synthase (*chs*) promoter fragment that is extremely active in ovules (Koes et al., 1990; Ylstra et al., 1994). Transgenic plants expressing the *chs–GUS* construct (Ylstra et al., 1994) were crossed with transgenic plant T46008. From the progeny, plants were selected that possessed the T46008 phenotype and expressed the *GUS* reporter gene in ovules (data not shown). In these plants, a high level of GUS activity was found in the ovulelike structures present on the sepals, whereas no GUS activity was detected in the sepal itself, as shown in Figures 2G and 2H. This demonstrates that *chs* promoter activity in ovulelike structures of T46008 sepals is similar to that in ovules.

Microscopic Analysis of T46008

To study the nature and development of the ovulelike structures on the sepals of transgenic plant T46008, both light microscopy and scanning electron microscopy were performed. Scanning electron microscopical analysis of the abaxial side of the sepals (Figure 3A) shows that the shape and organization of the epidermal and trichome cells of T46008 are identical to those found in wild-type sepals. Several differences were observed when the adaxial side of T46008 sepals (Figures 3B and 3C) was compared with that of the wild-type (Figure 3C). The cells of the inner surface of T46008 sepals are approximately five times smaller than the wild type (Figure 3C). In addition, instead of being organized in parallel lines, the cells have a random orientation. A comparison of these cells with placental cells (Figure 3D) shows striking similarities in shape, dimensions, and organization. The adaxial side of the sepals lacks trichomes, and several ovulelike structures are visible. The ovulelike structures are connected to the sepal inner epidermis by a structure that is comparable to the funiculus; it is also possible to distinguish a micropylar region, as shown in Figure 3E. Occasionally, at the base of the sepal close to the receptacle, the ovulelike structures occurred on epidermis (Figures 3B and 3F) closely resembling the morphology and organization of wild-type placenta, as shown in Figure 3D. Ovulelike structures sometimes developed on the outer side of the tube of the flower (Figures 3G and 3H); the cells of the tube surrounding these ovulelike structures are morphologically unaltered and are organized in parallel lines, as in the wild type (compare Figures 3H and 3I).

To determine the identity of the T46008 ovulelike structures that developed on the sepal, histological sections were made and analyzed by light microscopy. This analysis indicated that the ovulelike structures on the sepals of the transgenic plants (Figures 4D and 4E) are similar to wild-type ovules (Figure 4B). They resemble an ovule closely, including an epidermal cell layer, an integument, an embryo sac, and a funiculus connecting the ovule to the sepal. It is possible to identify the polar nuclei and the two synergid cells on the micropylar side of the embryo sac (Figure 4F), just as in the wild-type ovule. A longitudinal section of a sepal showed that the inner epidermal cells are smaller when compared with wild-type sepals (Figure 4A), especially those close to the ovulelike structures (indicated by an arrow in Figure 4C), and confirmed the results of scanning electron microscopic analysis (Figure 3C).

Expression of Other Petunia MADS Box Genes in T46008 Plants

To study the effect that ectopic *FBP11* expression has on the expression of the petunia MADS box genes *FBP11*, *FBP7*, *FBP1*, *FBP6*, and *pMADS3* (Angenent et al., 1992, 1993, 1995; Tsuchimoto et al., 1993), RNA gel blot analyses were performed (Figure 5). *FBP11* is expressed in all vegetative and flower tissues of T46008, whereas in W115 it is only expressed in whorl

Figure 3. (continued).

(A) Abaxial side of a T46008 sepal with trichomes.
(B) View of the adaxial side of a T46008 sepal on the right side. There is a placenta-like structure and several ovulelike structures indicated by an arrowhead.
(C) Adaxial side of a T46008 sepal. No trichomes are present, and the cells are very small in comparison with the wild type. At lower right, an ovulelike structure is visible. At upper left, the adaxial side of a W115 sepal is shown.
(D) A W115 placenta. Some of the ovules have been carefully removed to show the placental cell shape and organization.
(E) Ovulelike structure on a T46008 sepal.
(F) Ovulelike structure on the adaxial side of a T46008 sepal.
(G) View of a T46008 petal tube on which one ovulelike structure has developed.
(H) Detailed view of **(G)** showing an ovulelike structure on the tube of the petal.
(I) View of the tube of a W115 petal.
fu, funiculus; mp, micropyle; ov, ovule; ov*, ovulelike structure; pl, placenta; SE, sepal; T, tube; tr, trichome.

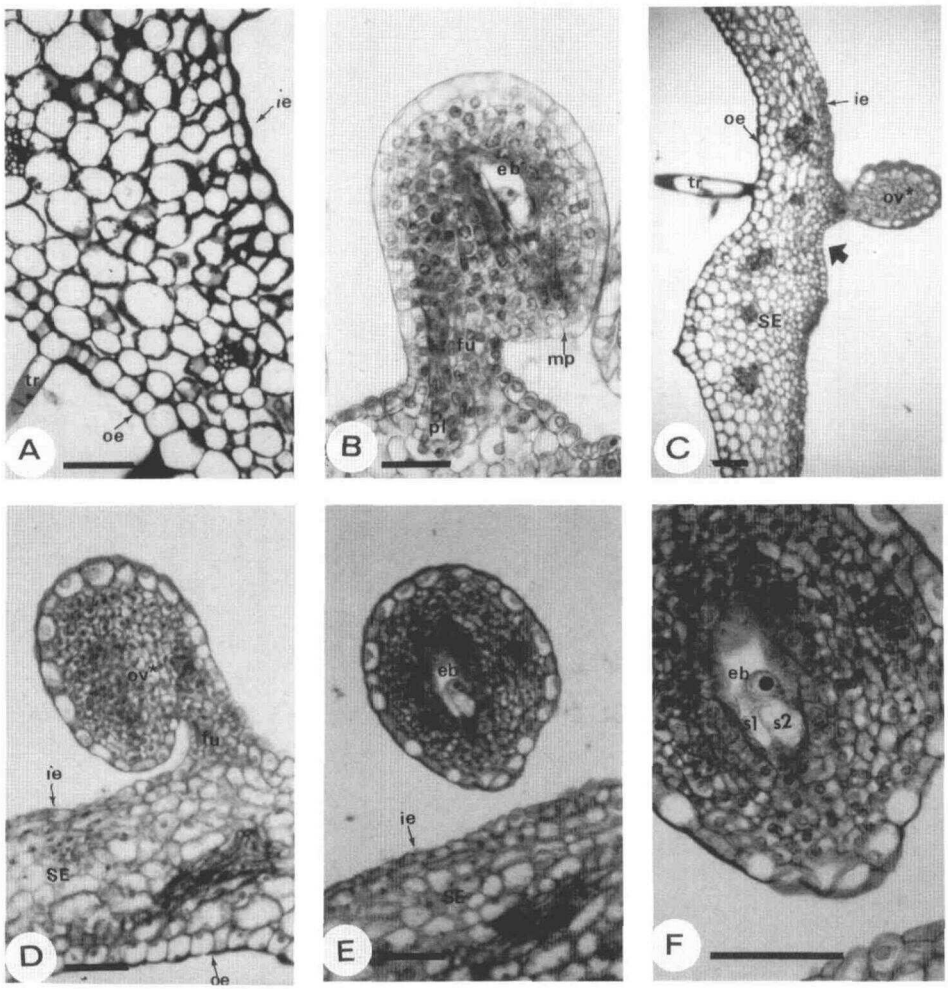

Figure 4. Microscopic Analysis of Sepals from T46008.

(A) Longitudinal section of a sepal from W115.
(B) W115 ovules.
(C) View of a T46008 sepal. The arrow indicates epidermal cells close to the ovulelike structures.
(D) and **(E)** T46008 ovulelike structure on a sepal; two different sections of the same ovulelike structure are shown.
(F) Embryo sac of one ovulelike structure on the sepal of T46008.
eb, embryo sac; fu, funiculus; ie, inner epidermis; mp, micropyle; oe, outer epidermis; ov*, ovulelike structure; pl, placenta; SE, sepal; s1 and s2, synergid cells; tr, trichoma. Bars = 0.2 mm.

4. Hybridization using the closely related ovule-specific *FBP7* cDNA as a probe revealed the same expression pattern in both the wild type and T46008: expression was only observed in carpels and not in other floral organs. No differences were observed in the expression of the petunia B-type MADS box gene *FBP1* between T46008 and W115. We were particularly interested in the expression of C-type MADS box genes in the transgenic plants to determine whether C-type gene expression is required for ovule development. The petunia genes *FBP6* and *pMADS3* are putative petunia C-type genes, being highly homologous to the Arabidopsis class C gene *agamous* (*ag*; Yanosky et al., 1990). Similar to *ag*, both petunia genes were found to be expressed in whorls 3 and 4 and at low levels in mature wild-type ovules (data not shown). In T46008, the expression patterns of *pMADS3* and *FBP6* in whorls 3 and 4 were unaltered with respect to the wild type. However, we observed that *FBP6* is also weakly expressed in sepals and that both *pMADS3* and *FBP6* are expressed in petals (Figure 5).

DISCUSSION

Ectopic *FBP11* Expression in Transgenic Plants Determines Ovule Development on Sepals and Petals

In this study, we investigated whether the ovule-specific *FBP11* gene is involved in determining ovule identity in petunia. To study *FBP11* activity, we made a CaMV 35S–*FBP11* chimeric gene enabling ectopic *FBP11* gene expression. Transgenic plants expressing *FBP11* at high levels developed ovulelike structures on sepals and to a lesser extent on petals. Histological analyses by light microscopy and scanning electron microscopy showed that the ovulelike structures that developed on the sepals had cells of the same shape and size as wild-type ovules. We also showed that sepal-located ovulelike structures in the *FBP11*-overexpressing genetic background exhibited *chs*-driven GUS activity similar to that found in wild-type ovules.

The presence of ovulelike structures on the sepals is accompanied by a transformation of the sepal inner epidermis into placental tissue. However, the development of placenta-like tissue does not seem to be a prerequisite for the determination of ovule identity because ovules were also formed on petals without any change in epidermal cell organization.

The development of ovulelike structures in whorl 1 was described in mutants obtained by ectopic expression of *ag* in Arabidopsis (Mizukami and Ma, 1992) and *BAG* (the Brassica homolog of *ag*; Mandel et al., 1992). These results showed that *ag* might be involved in the induction of genes regulating the formation and development of ovules. However, it has been observed that the Arabidopsis *apetala 2 ag* double mutant developed ovulelike structures in whorl 1 (Bowman et al., 1991). This suggests that *ag* itself is not necessary for the determination of ovule identity.

Many reports have been published about C-type gene expression in Arabidopsis, and as in several other plants, C-type genes are thought to determine the identity of whorl 4 meristem into pistil primordia (Mandel et al., 1992; Mizukami and Ma, 1992; Bradley et al., 1993; Tsuchimoto et al., 1993). However, until now, no genes have been isolated that induce the formation and development of ovule primordia. In Arabidopsis, the *BELL1* gene seems to be important for ovule development. However, this gene does not seem to determine the identity of ovule primordia because, even in the most severe *bell* mutant, the initial development of ovule primordia is normal. Only at later stages is the development of the integuments disturbed and carpelloid structures rather than integuments are formed (Robinson-Beers et al., 1992; Modrusan et al., 1994; Ray et al., 1994). In contrast to *bell*, in the *FBP11* cosuppression plants, ovule primordia are completely replaced with stigma–style structures (Angenent et al., 1995). In addition, our studies have shown that ectopic expression of *FBP11* determines ovulelike structure formation on sepals and petals. Taken together, our results suggest that *FBP11* is an ovule identity gene.

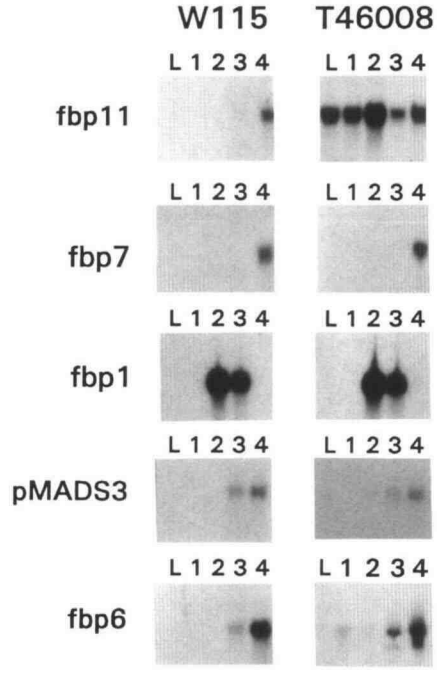

Figure 5. Expression of *FBP11* and Other MADS Box Genes in T46008 and W115.

Gel blot analysis of RNA isolated from W115 (left) and T46008 (right) using the *FBP11* cDNA (3′; 0.65 kb), *FBP7* cDNA (3′; 0.55 kb), *FBP1* cDNA (3′; 0.7 kb), *pMADS3* cDNA (3′; 0.6 kb), and *FBP6* cDNA (3′; 0.65 kb) as probes. Lanes L contain leaves; lanes 1, sepals; lanes 2, petals; lanes 3, stamens; lanes 4, carpels.

Interaction between *FBP11* and Other MADS Box Genes in Petunia

In this study, we analyzed the expression of other petunia MADS box genes in the transgenic plants ectopically expressing *FBP11*. Surprisingly, the ovule-specific MADS box gene *FBP7*, which is highly homologous to *FBP11*, was not induced by expression of *FBP11*. The presence of ovules on sepals and petals in the absence of *FBP7* expression suggests that ectopic expression of *FBP11* alone is sufficient to determine ovule development in whorls 1 and 2. The role of *FBP7* and the interaction between *FBP11* and *FBP7* will be the subjects of future studies.

In transgenic plants, the expression of *pMADS3* in whorl 1 and of *FBP6* in whorls 1 and 2 was observed. This could have been the consequence of a partial suppression of A-type genes by *FBP11*, resulting in the ectopic expression of the C-type genes in whorls 1 and 2. The low-level expression of C-type genes in whorls 1 and 2 of T46008 is in accordance with the changes of corolla size and could also be responsible for the

conversion of the sepal inner epidermal cell layer into placenta-like tissue. Expression of C-type genes may be required for the development of ovules in whorls 1 and 2 of transgenic plants. However, ectopic expression of *pMADS3* or *FBP6* did not result in ovule development on sepals and petals (Tsuchimoto et al., 1993; L. Colombo, unpublished data). In *blind*, a putative A-type gene petunia mutant, both *pMADS3* (Tsuchimoto et al., 1993) and *FBP6* (L. Colombo, unpublished data) are highly expressed in sepals and petals, without any sign of ovule development in whorls 1 and 2. Taken together, our results show that the petunia *FBP11* gene represents a new and important gene that is necessary for the induction of ovule development.

Extension of the ABC Model to an ABCD Model

The ABC model proposed by Coen and Meyerowitz (1991) describes the action and interactions of three classes of homeotic genes that determine the identity of the four floral whorls: sepals, petals, stamens, and carpels (Figure 6A). In light of our results, we believe that the ovule can be considered an independent whorl 4B floral organ whose induction and development are regulated by a specific class of MADS box genes. Previously, we hypothesized that the petunia flower consists of five whorls, each originating from distinct domains of the floral apex (Angenent et al., 1994). In transgenic plants in which *FBP2* expression is inhibited, carpels are formed without the formation of ovules and placenta (Angenent et al., 1994). Moreover, in transgenic plants in which *FBP1* expression is inhibited, ovules develop in the absence of a carpel (Angenent et al., 1993). These observations strongly suggest that carpel and ovule/placenta initiation and development can occur independent of each other. This agrees with palaeobotanic studies showing that the pteridosperms, the most likely progenitors of angiosperms, produced seeds from naked ovules. Furthermore, gymnosperms lack carpels, and free ovules develop on a sporophyll (Gifford and Foster, 1989).

We suggest that ovule induction in whorl 4B is under the control of a new class of MADS box genes, namely, class D genes, of which *FBP11* is a representative. Expression of class D genes is necessary for the correct determination of ovule identity and can be regarded as an inducer of ovule formation. At least in petunia, the ABC model might be extended to an ABCD model (Figures 6A and 6B) describing flower organogenesis as a consequence of the activities of four distinct classes of genes instead of three, as proposed in the ABC model. Together, the ABCD functions specify the identities of sepals, petals, stamens, carpels, and ovules.

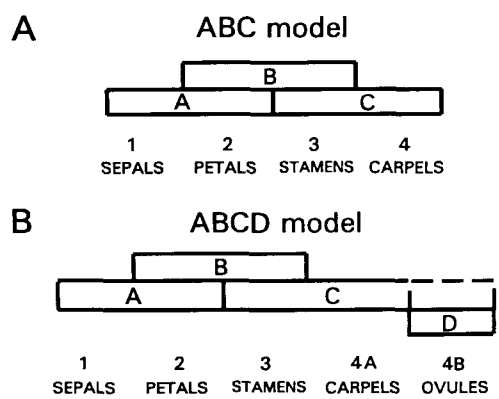

Figure 6. Models for Control of Organ Identity.

(A) The combinatorial ABC model according to Coen and Meyerowitz (1991). The identity of the four floral whorls has been determined by the action and interaction of three classes of genes. Each class of gene is active in two adjacent whorls. Class A genes specify sepals (whorl 1), A and B result in petals (whorl 2), B and C combined determine stamens (whorl 3), and class C genes lead to the formation of carpels (whorl 4).

(B) New extended model in which the D gene function is included. The D function determines the identity of ovules (whorl 4B). The involvement of C genes in ovule development is unclear and is indicated by a dashed line in whorl 4B. In petunia, *FBP6* and *pMADS3* belong to class C and *FBP11* to class D.

METHODS

Plant Material

The *Petunia hybrida* line W115 and transgenic plants were grown under normal greenhouse conditions.

Construction of Binary Vector and Plant Transformation

To facilitate cloning of the *FBP11* (for floral binding protein) cDNA under the control of the double enhancer cauliflower mosaic virus (CaMV) 35S promoter, new restriction sides were generated by polymerase chain reaction. The *FBP11* cDNA was amplified using a 5′ primer (5′-GTGCCATGGGGAGAGGAAAGATAGAG-3′) (Isogene Science, Maarssen, The Netherlands) that hybridized with *FBP11* sequences surrounding the ATG translation start site (underlined) and a 3′ primer (5′-GAGCAGATCTCTTTCTCCAGCGCCC-3′) (Isogene Sciences) that hybridized with *FBP11* sequences surrounding the translation stop site (underlined). The 5′ primer contains a NcoI recognition site, and the 3′ primer contains a BglII recognition site. After being sequenced, the amplified *FBP11* fragment was inserted as a NcoI-BglII fragment into the binary vector pCPO31. This binary vector was derived from pPCV708, as described by Florack et al. (1994), and contains three expression cassettes with a multiple cloning site between the left and right T-DNA borders. The cDNA was cloned between a modified CaMV 35S promoter containing a tandem duplication of 250 bp upstream of the TATA element and the nopaline synthase terminator sequence. The chimeric construct was transferred via Agrobacterium GV3101 to petunia variety W115 using the standard leaf disc transformation method

(Horsch et al., 1985). Regeneration of transformants was performed as described by van Tunen et al. (1989).

DNA and RNA Gel Blot Analyses

Plant DNA was isolated from petunia leaves according to Koes et al. (1986), and total RNA was isolated from leaves or mature floral tissues according to Verwoerd et al. (1989). Ten micrograms of DNA was digested with HindIII, electrophoresed, and blotted onto Hybond N^+ membranes (Amersham). For RNA gel blot analysis, 10 μg of total RNA was denatured by glyoxal (1.5 M) prior to electrophoresis. 3′ Terminal fragments of *FBP1* (0.6 kb), *FBP6* (0.73 kb), *FBP7* (0.5 kb), *FBP11* (0.78 kb), and *pMADS3* (0.6 kb) were radioactively labeled by random oligonucleotide priming (Feinberg and Vogelstein, 1984). Blots were hybridized as described by Angenent et al. (1992) and washed with 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 60°C for 20 min (three times) prior to autoradiography.

Microscopy

Sepals of T46008 and sepals and ovary of W115 were fixed for a minimum of 3 hr in 5% gluteraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The material was then rinsed four times for 15 min in 0.1 M sodium phosphate buffer, pH 7.2, and twice in distilled water for 10 min. The material was then dehydrated using a series of alcohol solutions (70, 90, and 100%) for 1 hr each. The material was embedded in (hydroxy-ethyl) methacrylate Technovit 7100 according to the protocol of the manufacturer (Kulzer Histo-tec, Wehrheim, Germany). After embedding, the material was placed in a mold, and 1 mL of Hardener II was added per 15 mL of Technovit, resulting in a polymerization reaction. The Technovit blocks were sectioned by microtome. Sections (15 μm) were stained for 1 min with a solution of 1% toluidine blue in distilled water.

For cryoscanning electron microscopy, the samples were mounted on a stub and subsequently frozen in liquid nitrogen. The samples were coated and observed as described in Angenent et al. (1995).

To detect β-glucuronidase (GUS) activity, sepals of the transgenic plant were stained according to the method described by Jefferson et al. (1987). The *GUS* reporter gene driven by the chalcone synthase (*chs*) promoter in transgenic petunia plants is active in various floral tissues (petals and stamens); however, extremely high *GUS* activity was observed in ovules (Koes et al., 1990).

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