# Temporal and Spatial Expression of MADS Box Genes, *FBP7* and *FBP11*, During Initiation and Early Development of Ovules in Wild Type and Mutant *Petunia hybrida*

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Received: July 12, 2000; Accepted: October 5, 2000

***Abstract:*** The temporal and spatial distribution of the Petunia Floral Binding Proteins 7 and 11 (FBP7/11) were determined immunocytochemically during ovule initiation and development. In wild type plants, FBP7/11 were first detected in the placenta before ovule primordia were formed. At ovule primordium stage, FBP7/11 levels increased in the placenta and appeared in ovule primordia at the sites where integument primordia developed. At the megagametogenesis stage, FBP7/11 were present at high levels in the placenta, funicle and integument, but not in the nucellus or gametophyte. Transgenics with cosuppression of FBP7/11 formed normal ovule primordia on the placenta from which both normal ovules and carpel-like structures developed. The amount of FBP7/11 was low in the ovules and undetectable in the carpel-like structures. Plants with ectopic expression of FBP7/11 developed normal ovules on the placenta and, in addition, ovule- and carpel-like structures on sepals. Placental and sepal ovules showed the same labeling pattern as observed in wild type ovules. FBP7/11 levels were, however, low or undetectable in the carpel-like structures. The results indicate that FBP7/11 only have indirect roles in ovule primordium initiation. However, at least small quantities are needed for proper ovule differentiation. Thus, the amount of FBP7/11 is related to the type of development after primordium formation, i.e., towards the formation of real ovules or carpel-like structures.

***Key words:*** *Petunia*, ovule development, immunocytochemistry, FBP7/11, MADS box genes.

***Abbreviation:***
FBP: Floral Binding Protein

## Introduction

Ovules are specialized structures, derived from the placenta in the ovary. They produce the female gametophytes and nurse the developing embryos after fertilization. The morphology and cytology of angiosperm ovules have been studied in a large number of species (Bouman, 1984[3]; Reiser and Fisher, 1993[10]; Willemse and van Went, 1984[17]), including *Petunia* (Angenent et al., 1995[1]; Lee et al., 1997[9]; van Went, 1970[13]). Usually, an ovule consists of three basic structures: the nucellus (enclosing the megagametophyte, that is the embryo sac), one or two integuments and a funicle. In several plant species, such as *Petunia*, the nucellus cells covering the embryo sac degenerate before the embryo sac reaches maturity. Hence, the embryo sac is in direct contact with the inner integument. In *Petunia*, the innermost layer of the integument differentiates into an endothelium, which is thought to be involved in the nutrition of the female gametophyte (Bouman, 1984[3]; Willemse and van Went, 1984[17]).

During the past years much has been learned about the molecular processes of flower development. Many MADS box genes, which play essential roles in determining meristem and floral organ identity, have been isolated and characterized (Angenent et al., 1992[2]), and the ABC model has been proposed to explain the genetic control of flower morphogenesis (Coen and Meyerowitz, 1991[5]). More recently, progress has been made in understanding the molecular control of ovule development (Schneitz et al., 1998[11]; Villanueva et al., 1999[15]). In *Petunia hybrida*, two ovule-specific MADS box genes, *Floral Binding Protein (FBP) 7* and *11*, have been molecularly characterized (Angenent et al., 1995[1]; Colombo et al., 1995[6]). The protein products, FBP7 and FBP11, of these genes are putative transcription factors with a MADS box DNA binding domain (Yanofsky et al., 1990[19]). Their overall amino acid sequences share ~90% identity. *In situ* mRNA hybridization experiments reveal that *FBP7* and *FBP11* are exclusively expressed in the centre of the gynoecium before ovule primordium initiation and, thereafter, in the developing ovules (Angenent et al., 1995[1]). After fertilization, *FBP7/11* are expressed in the developing seed coat (Colombo et al., 1997[7]). *FBP7* and *FBP11* are considered to belong to a new class, class D, of floral homeotic genes, supplementing the ABC model for floral organ development (Colombo et al., 1997[7]).

Transgenic plants with ectopic *FBP11* expression form normal ovules on the placenta and, in addition, ovule-like structures on sepals and rarely on petals. Transgenics with *FBP7/11* cosuppression show a homeotic transformation of ovules into carpel-like structures (Angenent et al., 1995[1]). Mildly cosuppressed plants exhibit carpel-like structures at the top of the pla-

Plant biol. 2 (2000) 693–702

ISSN 1435-8603

centa, whereas the remaining part of the placenta forms normal-looking ovules. These ovules can be fertilized but embryo formation becomes arrested due to the premature degeneration of the endothelium. In severe cosuppression plants most of the ovules are replaced by carpel-like structures (Colombo et al., 1997[7]). Based on these observations, it was suggested that *FBP7* and *FBP11* play key roles in the determination of *Petunia* ovule identity and seed development, although protein studies had not yet been performed. Recently, Wittich et al. analysed the post fertilization development of *Petunia* seeds and demonstrated that *FBP7/11* proteins are present in the cell nuclei. This nuclear position is in agreement with their supposed role as transcription factors (Wittich et al., 1999[18]).

In the present research we investigate ovule development in both wild type and transgenic plants and analyse the temporal and spatial distribution of FBP7/11 to understand the role of FBP7/11 in ovule development.

## Materials and Methods

### *Plant material*

Three types of *Petunia hybrida* plants were used: the wild type (WT) variety W115; its FBP11 ectopic expression transgenic T46008, described by Colombo et al. (1995[6]); and its FBP7 and -11 cosuppression transgenic T27017, described by Angenent et al. (1995[1]). In the ectopically expressing plants, the FBP11 gene was under the control of a strong cauliflower mosaic virus (CaMV) 35S promoter, resulting in ovule formation on sepals. In the cosuppressed plants, the full-length FBP11 cDNA was fused in the sense orientation behind the CaMV35S promoter (Angenent et al., 1995[1]). The plant used in this study has mild cosuppression, often showing a few carpel-like structures on the upper part of the placenta.

Plants were grown in a phytotron at 20 °C, with 16 h light and 8 h darkness. As described in Angenent et al., 1995[1], both the transgene and the endogenous genes are suppressed in the cosuppression line. This is a general phenomenon that we have observed in all our cosuppression lines.

### *Sample preparation*

Ovaries were dissected from the flowers of wild type and transgenic plants and cut in half. Sometimes carpels were removed. In addition, parts of sepals with ovule-like structures were dissected out. Further processing was as described by Wittich et al. (1999[18]). In short, samples were rapidly freeze-fixed in liquid propane, the frozen samples were freeze-substituted in acetone containing 1% glutaradehyde in a freeze-substitution machine, further dehydrated through an acetone–ethanol series, and infiltrated and embedded in butyl methyl methacrylate (BMM). The embedded samples were polymerized under UV light at −20 °C for 40 h. Sections of 3 µm thickness were prepared for immunolabelling, and 1–2 µm thick sections were stained with 0.1% toluidine blue in water.

For scanning electron microscopy, ovaries and sepals were dissected and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.2) for 12 h at 4 °C. After rinsing in PBS for 3 h, they were dehydrated in an ethanol series, and critical point-dried using a Balzer Union CPD 020. The samples were mounted on stubs, sputter-coated with gold, and observed with a JSM-5200 scanning microscope. Digital images were collected with an Orion frame grabber.

### *Immunocytochemistry*

The immunological detection of FBP7/11 is described by Wittich et al. (1999[18]). Sections were rinsed in acetone to remove BMM, blocked with 0.1 M hydroxylammonium chloride for 5 min, and blocked again with 1% bovine serum albumin (BSA) for 30 min. Sections were incubated overnight at 4 °C with an antibody raised in rabbit against the C-terminal part of FBP11. The polyclonal antibody also recognizes FBP7 because of the high levels of similarity between the polypeptide sequences (Colombo et al., 1997[7]). After rinsing in PBS supplemented with 0.1% acetylated BSA, the sections were incubated with a goat anti-rabbit antibody conjugated with alkaline phosphatase (GaR-AP) for 1.5 h at 37 °C. The sections were rinsed and then incubated with a mixture of dimethylformamide (DMF), nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at pH 9.25, at room temperature for 90 min. Images of the purple-blue reaction product were recorded with a Sony CCD video camera on a Nikon Optiphot microscope.

Treatment with pre-immune serum and omission of the first antibody were used as controls. Another control was done by incubating sections from wild type sepals, of which is demonstrated that they do not contain the mRNA of *FBP7* and *FBP11* (Colombo et al., 1997[7]).

Labeling densities on the various sections were compared by analysis of digital images. These images were obtained under fixed conditions of section thickness, labeling time, exposure time and illumination. Grey levels of unlabeled and labeled tissues were determined for at least five sections per treatment.

### *Clearing of ovules*

Ovules were removed from placenta and sepals and fixed in a mixture of formalin, acetic acid and 70% alcohol (FAA, 5 : 5 : 90) for 24–48 h. After dehydration through an ethanol series, they were cleared through a 3 : 1, 1 : 1, 1 : 3 and 100% ethanol : methyl salicylate series, 15 min for each step (Stelly et al., 1984[12]). The cleared ovules were observed with a Nikon Optiphot microscope using Normaski optics. Digital images were made with a Sony CCD video camera.

## Results

### *Ovule development in wild type and transgenic* Petunia

Ovules of wild type and transgenics were compared with respect to structure and development. Ovules were only taken from the middle region of the ovaries because the ovules in the upper part developed earlier than those in the basal part of the ovary. A comparative table of ovule development is presented in Table **1**. Whereas the cosuppressed and WT plants show a similar pattern of development relative to flower bud size, the ectopic expression plants exhibited reduced bud lengths because of limited petal development. At anthesis, all the ovules within one ovary contained mature embryo sacs.

**Table 1** Ovule development in wild type *Petunia*, the *FBP11* ectopic expression mutant T46008 and the *FBP7/11* cosuppression mutant T27017 in relation to flower bud length

| Ovule stages | Morphological description of placenta ovules | Bud length (mm) | | | Ovary stages* |
|---|---|---|---|---|---|
| | | Wt | T46008 | T27017 | |
| 0 before primordia | smooth placental epidermis | 2–3 | <2 | 2–3 | stage 4–5 |
| 1 ovule primordium | ovular primordia arise | 3–6 | 2–3 | 3–6 | stage 6 |
| 2 archespore | nucellus and archespore develop, integument initiates | 6–8 | 3–5 | 6–8 | stage 8–10 |
| 3 megasporocyte | ovule stalking, integument shorter than the nucellus, megasporocyte enlarges | 8–10 | 5–8 | 8–10 | |
| 4 meiosis | ovules anatropous, integument covers the nucellus, megasporocyte undergoes meiosis | 10–13 | 8–10 | 10–13 | stage 11 |
| 5 megaspore | non-functional megaspores degenerate | 13–15 | 10–12 | 13–15 | stage 12 |
| 6 megagemetogenesis | functional megaspore undergoes 3 successive mitosis forming coenocytic megagametophyte, nucellus degenerates | 15–55 | 12–22 | 15–55 | stage 13 |
| 7 cellularization | development of egg apparatus, central cell and antipodals | 55–65 | 22–32 | 55–65 | stage 14 |
| 8 mature ovule | ovule with a seven-celled embryo sac at anthesis | >65 | >32 | >65 | stage 15 |

*According to the classification by Angenent et al. (1995[1])

### *Ovule development in wild type* Petunia

The morphological development of *Petunia* ovules is shown in Figs. **1A–D** and the anatomy in Figs. **1E–L**. Before the onset of ovule primordium formation (stage 0), the placenta had a smooth epidermis without extending ovule primordia. The cells of the outer layers of the placenta were filled with cytoplasm and contained large nuclei (Fig. **1E**). At stage 1, ovule primordia arose from the epidermal and subepidermal cells of the placenta (Figs. **1A,F**) and consisted of cells with much cytoplasm. The cells of the central region of the placenta had large vacuoles. At stage 2 of ovule development, the apical region of the ovule primordia formed the nucellus. The subepidermal nucellus cell, directly below the apex, developed into the archespore. At stage 3, the ovule had formed a funicle and its integument primordia did not yet cover the nucellus (Figs. **1B,G**). The nucellus consisted of a single layer of elongated cells that covered the megasporocyte (Fig. **1H**). Hereafter, the ovules became anatropous (Fig. **1C**). The integument had enlarged and covered the nucellus completely (Fig. **1I**). At stage 4, the megasporocyte underwent meiosis resulting in a linear tetrad of megaspores. At stage 5, the three megaspores of the tetrad on the micropylar side degenerated, and that on the chalazal side became the functional megaspore (Fig. **1J**). At stage 6, the functional megaspore divided, forming a coenocytic megagametophyte whereas the nucellus degenerated leaving a layer of cell walls enclosing the embryo sac (Fig. **1K**). At stage 8, the mature ovule consisted of a funicle, a well-developed integument, and an embryo sac with egg apparatus, central cell, and three antipodal cells (Fig. **1L**). The placental cells were filled with starch granules when the ovules became mature.

### *Ovule development in transgenic* Petunia

Cosuppressed plants formed normal-looking ovules and carpel-like structures on the placenta (Fig. **2A**). Again, the process of ovule development started with primordium formation (Fig. **2B**) and for most primordia this was similar to that observed in the wild type (Table **1**). The primordia in the upper part of placenta, however, elongated greatly and developed into carpel-like structures (Fig. **2A**). Some primordia in the upper region of the placenta developed into "ovule-like" structures that contained an aberrant nucellus or an abnormal embryo sac (Fig. **2C**). The primordia in the middle and lower part of the placenta developed into normal-looking ovules consisting of a funicle, an integument and a well-developed embryo sac with starch granules (Fig. **2D**).

Flower development of the ectopically expressed plant deviated from that of the wild type and the cosuppressed plants. The sizes of the flower buds were smaller than those of the WT and cosuppression plants because petal development was severely disturbed (Table **1**). Ovule development on the placenta was similar to that observed in the wild type, however, ovules were also formed on the sepals. (Fig. **2E**, arrows). Whereas wild type sepals were characterized by trichomes (Fig. **2G**), the surface of the adaxial epidermis of T46008 sepals resembled the epidermis of the placenta, having a smooth appearance without trichomes and profiles of small cells (Fig. **2F**, see also Colombo et al., 1995[6]). In addition, the cells of the sepal showed a similar structure to those of the placenta and also contained starch granules. The "ovule" primordia on the sepal arose from the adaxial epidermis in the region where two sepals had fused. Some primordia developed into normal-looking ovules (Figs. **2H–J**), others exhibited changed development and either elongated and developed into carpel-like structures (Figs. **2E,K**) or formed intermediate structures (Fig. **2L**). Some ovules contained a well-developed embryo sac (Fig. **2M**), others contained nucellar-like cells in place of the embryo sac (Fig. **2N**) or only contained a collapsed nucellus (Fig. **2O**).

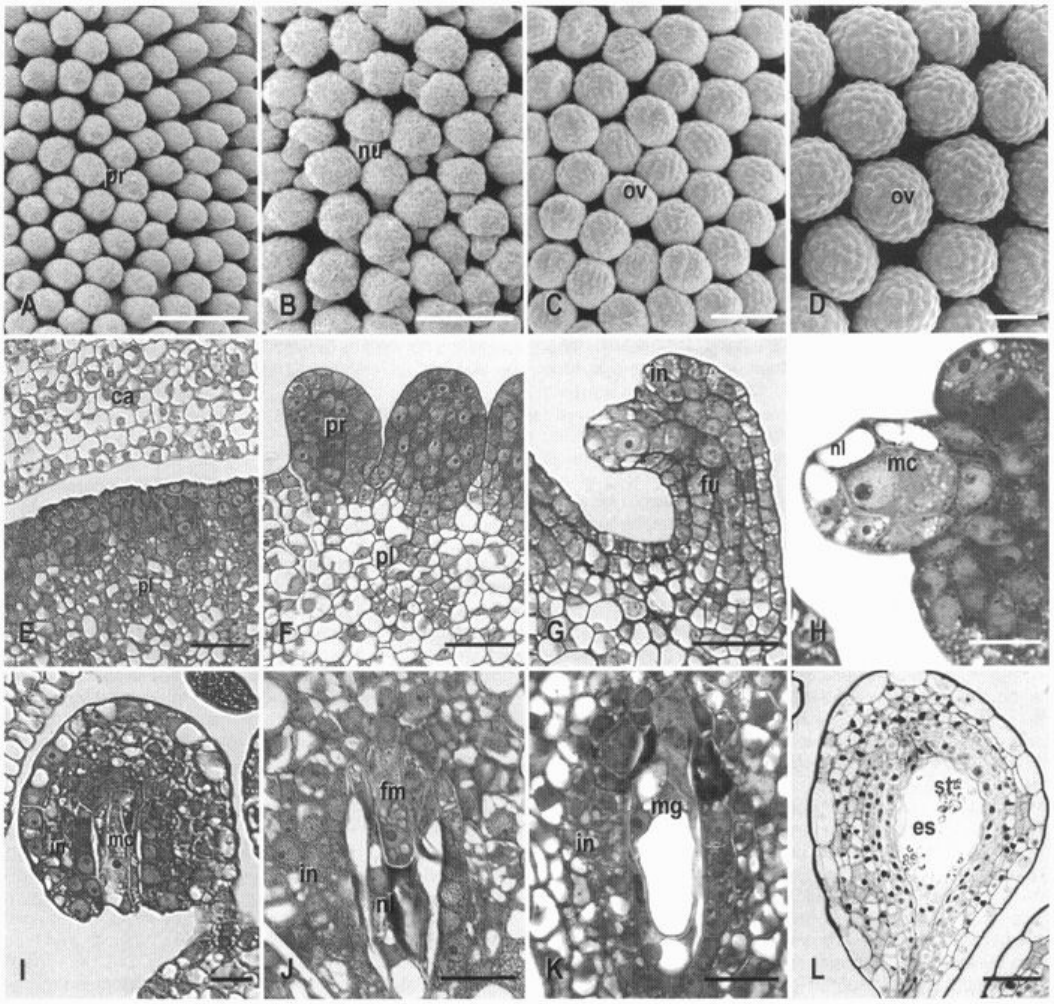


**Fig. 1** Ovule development in WT *Petunia* observed by scanning electron microscopy (**A–D**) and light microscopy (**E–L**). Sections were stained with toluidine blue. **A** Ovule primordia *(pr)* on the placenta at stage 1 (bud length 6 mm). **B** Bent ovules with elongated nucellus *(arrow – nl)* and initiation of integument at the base of the nucellus at early stage 3 (bud length 9 mm). **C** Anatropous ovules *(ov)* at the middle stage 3 (integument shorter than nucellus, bud length 10 mm). **D** Anatropous ovules *(ov)* at stage 6 (bud length 50 mm). **E** Detail of placenta *(pl)* before ovule primordium formation (stage 0). **F** Detail of ovule primordia *(pr)* formed on placenta *(pl)* (stage 1). **G** Overview of a bent ovule with developing megaspore mother cell (arrow) at early stage 3. *in* = integument, *fu* = funicle. **H** Detail showing integument *(in)* formation and the single cell layer thick nucellus *(nl)* covering the megaspore mother cell *(mc)*. **I** Anatropous ovule with elongated integument (*in*) and nucellus surrounding the mature megaspore mother cell *(mc)* at late stage 3. **J** Detail of ovule at stage 5, showing the degenerating nucellus *(nl)* surrounding the functional megaspore *(fm)*. *in* = integument. **K** Detail of ovule with developing megagametophyte *(mg)* at stage 6. The nucellus degenerates. **L** Mature ovule with a cellularized embryo sac *(es)* at stage 8, showing accumulation of starch grains *(st)* in the central cell. Bar size for **A–D** = 100 µm; **E–G** and **L** = 40 µm; **H–K** = 20 µm.

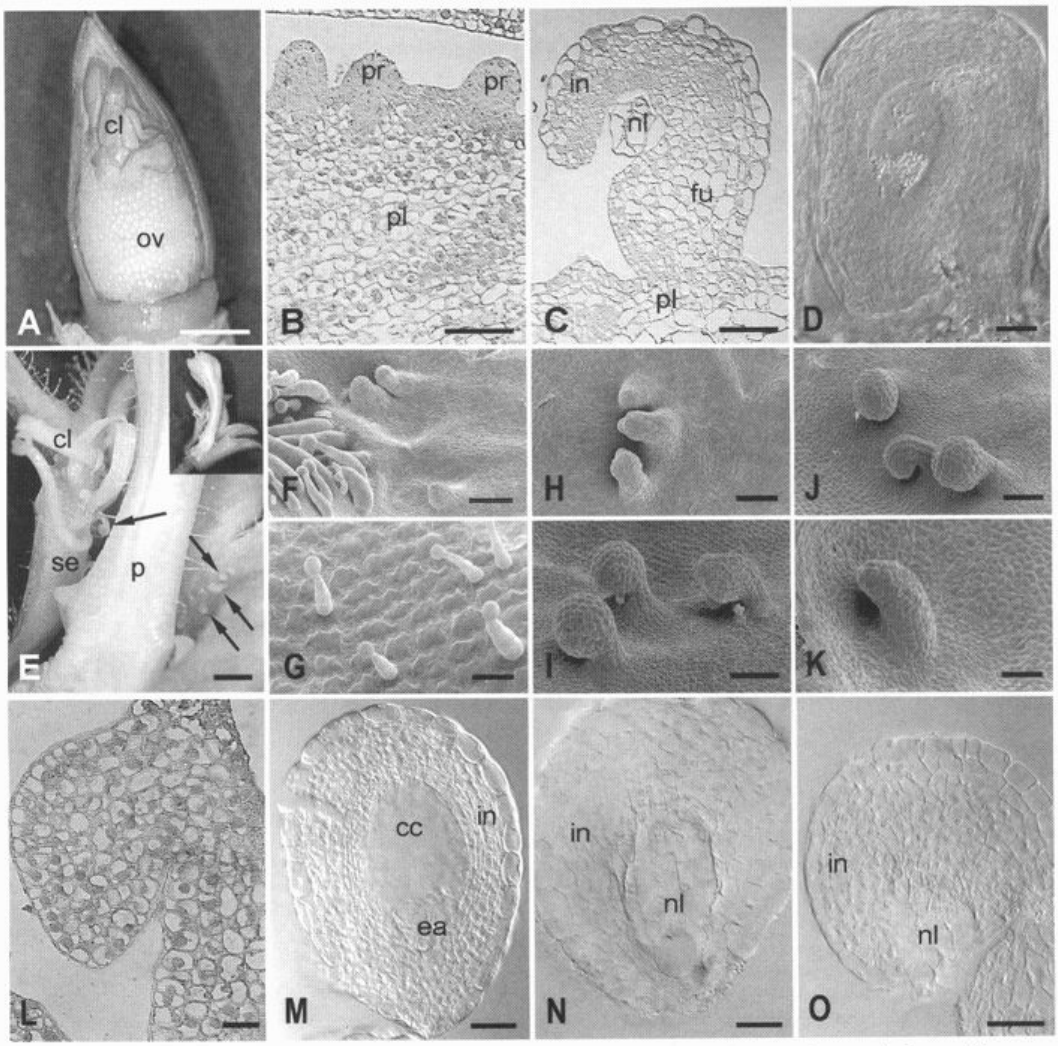


**Fig. 2** *Petunia* ovule development on the placenta of the *FBP7/11* cosuppression mutant T27017 (**A–D**) and on the sepals of the *FBP11* ectopic expression mutant T46008 (**E, F, H–O**) observed with light microscopy (**A–E, L–O**) and scanning electron microscopy (**F–K**). **A** Overview of the placenta with ovules *(ov)* after removing the carpel at the mature ovule stage. Note that normal ovules are formed in the middle and basal part of the placenta and that carpel-like structures develop at the distal part. **B** Detail of placenta *(pl)* with ovule primordia *(pr)* at stage 1. **C** An aberrant ovule at the mature stage with a malformed nucellus-like structure at the site of the embryo sac. *in* = integument; *nl* = nucellus; *fu* = funicle. **D** Overview of a normal ovule at the mature stage, showing a well-developed embryo sac with starch grains. (**E–O**) Ovule development on the sepals of the ectopic expression mutant T46008. **E** Detail of the basal part of a flower at the mature stage with the petal tube *(p)* surrounded by sepals on which ovules (arrows) and carpel-like structures *(cl)* are formed. The insert is the overview of the same flower with one sepal removed. **F** Detail of the adaxial epidermis at the fusion area of two sepals at stage 1 (bud length 3 mm), showing a hairy edge at the left hand side, and three ovule primordia. Note the placenta-like appearance of the surface of the sepal. **G** Detail of the adaxial sepal epidermis of the WT *Petunia*, showing trichomes. **H** Ovule-like structures on the sepal at stage 3 (bud length 5 mm), showing bent ovules with the nucellus only partly covered by the integument. **I** Developing anatropous ovules on a sepal at stage 4 (bud length 9 mm). Note that the nucellus of one ovule is still not covered by the integument (arrow). **J** Mature anatropous ovules on sepal at anthesis. **K** Aberrant primordium development at stage 3 (bud length 6 mm). The elongated structure lacks nucellus and integument and will probably develop into a carpel-like structure. **L** Light micrograph of section showing an ovule-like structure at the adaxial side of the sepal. (**M–O**) Cleared ovules from sepals of T46008 flowers at anthesis. **M** An ovule with normal embryo sac with egg apparatus *(ea)*. *cc* = central cell, and *in* = integument. **N** Ovule-like structure with nucellus-like cells *(nl)* in place of the embryo sac. *in* = integument. **O** Ovule with an integument *(in)* that does not cover the nucellus-like cells *(nl)*. Embryo sac is not present. Bar size for **A** and **E** = 1 mm; for **B, G** and **K** = 50 µm; for **C, M–O** = 25 µm; for **D** and **L** = 20 µm for **F, H–J** = 100 µm.

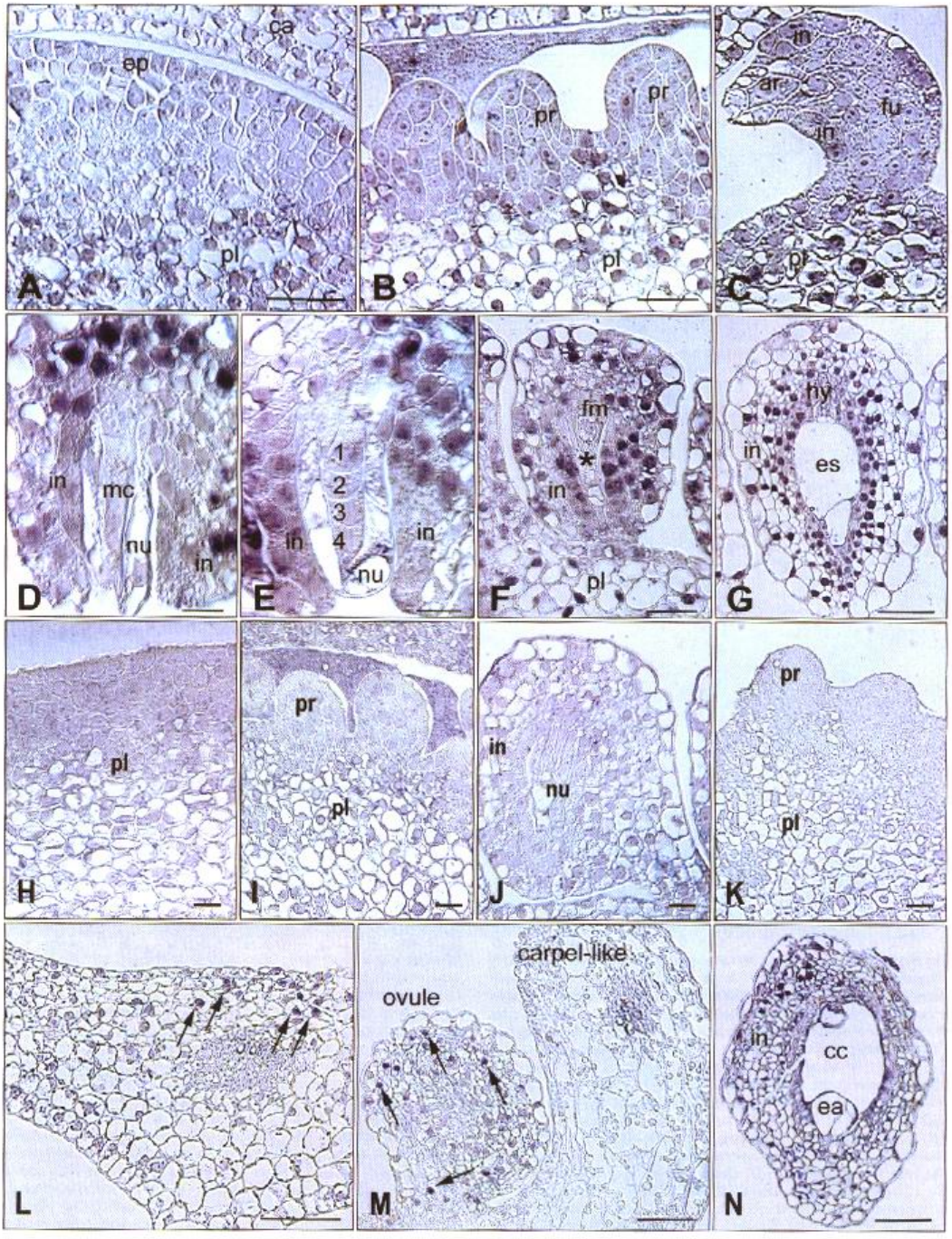
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Fig. 3

### *Immunocytochemical detection of FBP7/11 in developing ovaries of wild type and transgenic* Petunia

The distribution of the FBP7/11 proteins was determined from ovule initiation until ovule maturation in wild type *Petunia* (Figs. **3A–G**), in the *FBP7/11* cosuppression plants (Figs. **3H–K**), and in the *FBP11* ectopic expression plants (Figs. **3L–N**).

*Wild type*. At stage 0, that is before the initiation of wild type ovule primordia, a faint immunolabeling signal of FBP7/11 was observed in the nuclei of the epidermal and subepidermal cell layers of the placenta, and a clear signal was found in the centre of the placenta, especially in the nuclei (Fig. **3A**). With the formation of ovule primordia, i.e., stage 2, FBP7/11 labeling became more pronounced in nuclei of the placenta and, in addition, it appeared at a low level in nuclei of the ovule primordia (Fig. **3B**). The ovules at stage 2–3 clearly exhibited FBP 7/11 labeling in nuclei of the funicle and the integument primordia, but not in nuclei of the nucellus and archespore (Fig. **3C**). During meiosis, stage 4, the labeling became prominent in nuclei of the integument and chalaza (Fig. **3D**). After meiosis, late stage 4, the integument nuclei surrounding the nucellus were all well-labeled (Fig. **3E**). Most cells of the integument still showed FBP7/11 labeling after degeneration of the non-functional megaspores (stage 5, Fig. **3F**). At megagametogenesis, stage 6, the amount of FBP7/11 increased in the funicle and integument cells, but the degenerating nucellus and the enlarging coenocytic megagametophyte were again not labeled (data not shown). In mature ovules, stage 8, high levels of FBP 7/11 were again observed in funicle and integument (Fig. **3G**). During the various stages of ovule development, FBP7/11 were also observed in the nuclei of the placental cells (Figs. **3C,F**).

*Cosuppression plants*, showed very faint FBP7/11 labeling in the nuclei of the placenta before ovule primordium formation (Fig. **3H**). The level of FBP7/11 was less than 10% compared to the signal in wild type. This low level of signal was also observed in the nuclei of ovule primordia (Fig. **3I**). High levels of FBP7/11 were never seen during the later stages of ovule development (Fig. **3J**) but the signal remained above background level compared to the pre-immune serum control (Fig. **3K**).

The expression patterns of FBP7/11 in the ovary and placental ovules of the *ectopically expressing plants* were similar to those observed in the wild type, except that FBP7/11 were also detected in the carpels of the ectopic plant (data not shown). Expression of FBP7/11 were also found in the sepals, even before the formation of the sepal ovules, FBP7/11 were detected in the nuclei of the sepal cells, especially in epidermal and subepidermal cells on the adaxial side in the region where the primordia would arise (Fig. **3L**). At later stages, the immunolabeling signal was always present in the sepal ovules and ovule like structures (Fig. **3M**, left hand side, and N) but FBP7/11 were not observed in the carpel-like structures (Fig. **3M**, right hand side). As in the placental ovules, FBP7/11 were again found in the nuclei of the funicle and integument cells, but not in the nucellus or gametophytic cells (Fig. **3N**).

◀ **Fig. 3** Immunolocalization of FBP7/11 proteins on sections of wild type (**A, B, D, G** and **K**) *Petunia*, the *FBP7/11* cosuppression mutant T27017 (**H–J**), and the *FBP11* ectopic expression mutant T46008 (**C, E, F, L–M**). The NBT precipitate (blue signal) is detected by bright field microscopy. **A** Overview of WT placenta *(pl)* before ovule primordium formation, showing clear FBP7/11 signal in the nuclei of the vacuolated cells in the inner zone, and a low level in epidermal *(ep)* and subepidermal layers. co – Carpel. **B** Ovule primordia on WT placenta, showing clear FBP7/11 signal in the placenta *(pl)* and a low level in ovule primordia *(pr)*. **C** Ovule at archespore *(ar)* stage in T46008, showing high labeling in the placenta *(pl)* and low labeling in integument primordia *(in)* and funicle *(fu)* of the ovule. **D** WT ovule at meiosis, showing high labeling in the chalaza and integument *(in)*. Signal is absent in the nucellus cells *(nu)* and the diade *(mc)*. **E** Ovule at four-megaspore stage in T46008, showing increase of FBP7/11 signal in the integument *(in)* proceeding towards the micropyle. Signal is absent in the nucellus *(nu)* and tetrad (1–4). **F** Ovule at functional megaspore stage in T46008, showing FBP7/11 signal in the placenta *(pl)* and integument *(in)*. It was absent in the functional megaspore (fm), the three degenerating megaspores and the degenerating nucellus cells (asterisk). **G** Mature WT ovule, showing FBP7/11 signal in integument cells *(in)*, but not in the embryo sac *(es)* and hypostase *(hy)*. (**H–J**) Distribution of FBP7/11 signal during ovule development in the cosuppression mutant T27017. **H** Detail of placenta before ovule primordium formation in T27017, showing a very low level of FBP7/11 in the nuclei. **I** Ovule primordia on the placenta in T27017, showing a low level of FBP7/11 signal in both the placenta and primordia. **J** Ovule at mature megaspore mother cell stage, again showing low levels of FBP7/11 signal in integument and funicle. **K** Ovule primordia *(pr)* and WT placenta *(pl)* stained with pre-immune as a control, showing no FBP7/11 signal in the nuclei. (**L–N**) Distribution of FBP7/11 signal during ovule development on the sepals of the ectopic expression mutant T46008. **L** Cross section of young sepal of flower bud of T46008 before the formation of ovule primordia, showing clear FBP7/11 signal in the nuclei of the adaxial epidermal and subepidermal cells (arrows). **M** T46008 sepal with FBP7/11 signal in the cell nuclei (arrows) of an ovule-like structure (left hand side) but not in the carpel-like structure (right hand side). **N** A sepal ovule of T46008, showing FBP7/11 signal in the nuclei of the integument *(in)*, but not in the central cell *(cc)* or egg apparatus *(ea)*. Bar size for **A–C, F, G** and **N** = 25 µm; for **D** and **E** = 10 µm; for **H–K** = 20 µm; for **L** and **M** = 50 µm.

## Discussion

The development of ovules includes the following processes: primordium initiation, ovule identity specification, ovule differentiation, and identity maintenance. The present study is an analysis of the temporal and spatial distribution of the Floral Binding Proteins 7 and 11 during ovule initiation and development. Fig. **4** provides a schematic summary of the events and the simultaneous occurrence of FBP7/11.

### *Ovule primordium formation is independent of FBP7/11*

As the pattern of FBP7/11 is first expressed in the centre of the wild type placenta before ovule primordium formation, and then in its outer layers and in the developing ovule primordia, these might express the ability of the placental tissue to generate ovule primordia. The nuclear position of FBP7/11 is in agreement with its function as a transcription factor. Such a position has also been reported for other FBPs (Cañas et al., 1994[4]). The ectopically expressing plants also formed FBP7/11 in the adaxial epidermal and subepidermal cell layers of the young sepal and indeed on these sites ovule primordia were later formed. However, mild cosuppression mutants, which exhibit very low levels of FBP7/11 in the placenta, still showed ovule primordium initiation. Moreover, severe cosuppressed plants without any expression of FBP7/11 were also able to form ovule primordia (Angenent et al., 1995[1]). There-

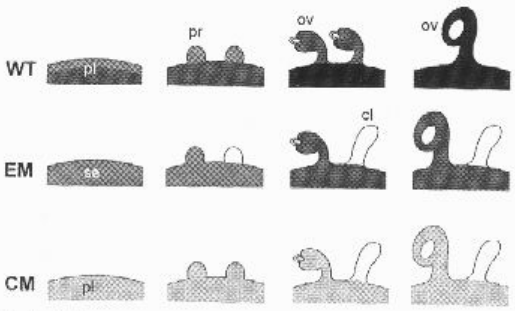


**Fig. 4** Schematic representation of FBP7/11 distribution in the placentae (*pl*), sepals (*se*), ovule primordia (*pr*), ovules (*ov*), and carpel-like structures (*cl*) of wild type *Petunia* (**WT**), the *FBP11* ectopic expression mutant T46008 (**EM**), and the *FBP7/11* cosuppression mutant T27017 (**CM**). The grey scale mimics the FBP7/11 levels determined by antibody-alkaline phosphatase detection from unlabeled (white) to well-labeled (black). **WT** FBP7/11 proteins are present in the placenta of WT and EM before ovule primordium initiation, become gradually detectable in the ovule primordia and accumulate in the developing ovules of wild type *Petunia*. **EM** FBP7/11 are present in sepals and developing sepal ovules of EM, but absent in the developing carpel-like structures. **CM** During ovule development in T27017 (CM), low levels of FBP7/11 are detected in the placenta, the ovule primordia and developing ovules, but absent in the carpel-like structures. Taking all this together, it is concluded that initiation of ovule primordia is independent of FBP7/11, low levels of FBP7/11 proteins play a key role in further ovule development and high levels are necessary for proper seed formation. The absence of FBP in primordia results in a lack of specification of *Petunia* ovule identity, and thus to the formation of carpel-like structures.

fore, it is likely that the initiation of ovule primordia does not depend on the presence of FBP7/11.

What then is the role of FBP7/11 in the sepals of the ectopically expressing plant at this stage? Colombo et al. (1995[6]) demonstrated that the ectopic expression of FBP11 promotes the expression of C-type genes in the sepals (see also Kater et al., 1998[9]). We propose that FBP11 might induce placenta-like characteristics in the adaxial tissues of the sepal. Then the sepals C-type genes induced by FBP11 trigger the placenta-like development of the sepals which thus attain the ability to form ovule primordia. We cannot rule out that FBP7/FBP11 can act as a C-function gene when misexpressed. However, FBP7/FBP11 and homologous genes in species, such as *Arabidopsis* (AGL11), are clearly in a different subgroup of MADS box genes from the AG-like genes. To our knowledge there is no report describing an ectopic C-function when a MADS box gene belonging to the AGL11/FBP7/11 subgroup is misexpressed. Furthermore, the ectopic ovules on the sepals and the ectopic expression of the C gene pMADS3 cannot be explained by the assumption that FBP7/11 simply act as a C function gene.

### *Ovule identity specification*

The immunolabelling results show that the FBP7/11 proteins accumulate at various levels in primordia and ovules. Low levels of FBP7/11 are sufficient for primordium specification towards ovule development, as observed in mild cosuppressed plants, but when FBP7/11 levels are below a certain threshold, primordia may only develop to carpel-like structures. This is seen in some primordia on the sepals of the ectopic expression plant, in some on the placenta in the mild cosuppressed plant, and in all primordia in severely cosuppressed plants. Thus, the level of the FBP7/11 relates to the fate of the primordia; i.e., specification of the identity of the primordia towards ovule development is uniquely accompanied by *FBP7/11* expression.

### *FBP7/11 play a role in ovule differentiation*

After the elongation of the ovule primordium, ovule differentiation proceeds with the initiation of integument primordia. FBP7/11 gradually accumulate in the developing integument and funicle of the placental ovules of the wild type and in the sepal ovules of the ectopic expression plant. Because both carpel-like structures on the sepals of the ectopic expression plant and the carpel-like structures in the ovary of the cosuppression plant did not contain FBP7/11 at detectable levels, whereas FBP7/11 were present in the sepal-borne ovules and ovule-like structures, it is assumed that FBP7/11 play an essential role in proper ovule differentiation and need to be present in sufficient amounts. But, again, high levels are not needed, as observed in the mild cosuppression plant.

Some ovule-like structures are arrested during their development, both on the sepals of the ectopic plant and on the placenta in the mild cosuppression plant, on the sepals. They exhibited lower levels of FBP7/11 than the well-developed ovule-like structures. This again indicates that the amount of FBP7/11 might be correlated with the extent of ovule differentiation, a phenomenon also observed in *Arabidopsis* (Western and Haughn, 1999[16]).

### *FBP7/11 have a function in the maintenance of ovular tissues*

Analysis of mature ovules of wild type and ectopic expression plants showed that FBP7/11 were present in the integument, chalaza and funicle, but undetectable in the nucellus and embryo sac. The high levels of FBP7/11 in the funicle, chalaza and integument might be required for maintaining the identity and function of those tissues. The nucellus is the only differentiated tissue that did not contain FBP7/11 and, indeed, it degenerates during the enlargement of the coenocytic megagametophyte whereas the funicle, chalaza and integument contribute to further seed development. Wittich et al. (1999[18]) recently suggested that FBP7/11 are needed for the maintenance and function of the integument-derived seed coat during seed development. They found that FBP7/11 could not be detected in the ovules of mild cosuppression plants. There was premature degeneration of the inner layer of the integument, the endothelium, which resulted in the arrest of endosperm and embryo development (Colombo et al., 1997[7]; Wittich et al., 1999[18]). The present study again points to a role for FBP7/11 in cell and tissue maintenance, but at the stage of ovule development low levels of FBP7/11 protein are likely sufficient for proper development.

In summary, the present studies reveal key roles played by FBP7/11 during ovule development. Together with the data obtained from studies on the role of FBP7/11 during seed development (Wittich et al., 1999[18]), it can be concluded that 1) ovule primordium formation is not directly dependent on the

presence of FBP7/11; 2) FBP7/11 are necessary for ovule identity specification and differentiation but high levels are not a prerequisite; 3) seed development is only successful when ovules accumulate high amounts of FBP7/11; and 4) FBP expression in the sepals demonstrates the placenta-like properties of the sepal resulting in ovule primordium formation and further development.

**Acknowledgements**

The project was financed by the EU (BIO4CT960390).

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Section Editor: M. Koornneef