Downregulation of Ovule-Specific MADS Box Genes from Petunia Results in Maternally Controlled Defects in Seed Development

Lucia Colombo,a,† John Franken,a Alexander R. Van der Krol,b Peter E. Wittich,c Hans J. M. Dons,a and Gerco C. Angenent*a

aDepartment of Developmental Biology, DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Droevendaalsesteeg 1, P.O. Box 16, 6700 AA Wageningen, The Netherlands
bDepartment of Plant Physiology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands
cDepartment of Plant Cytology and Morphology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

A maternally determined seed defect has been obtained by downregulation of the petunia MADS box genes Floral Binding Protein7 (FBP7) and FBP11. These genes have been previously shown to play central roles in the determination of ovule identity. Aberrant development of the seed coat and consequent degeneration of the endosperm have been observed in transgenic plants in which these two genes are downregulated by cosuppression. Analysis of the expression pattern of FBP7 and FBP11 and genetic analysis confirmed the maternal inheritance of the phenotype. The FBP7 promoter was cloned and fused to reporter genes. One of these reporter genes was the BARNASE gene for targeted cell ablation. Our results indicate that FBP7 promoter activity is restricted to the seed coat of developing seeds and that it is completely silent in the gametophytically derived tissues. The mutants used in this study provided a unique opportunity to investigate one of the poorly understood aspects of seed development: the interaction of embryo, endosperm, and maternal tissues.

INTRODUCTION

During floral organogenesis, five different types of organ primordia emerge from the floral meristem and differentiate into the floral organs that are organized in concentric whorls. From the outer to the inner whorls, these organs are sepals, petals, stamens, carpels, and, in the center of the flower, the placenta bearing the ovules. The placenta develops as a distinct meristematic region inside the petunia carpel (Angenent et al., 1995; Colombo et al., 1995). The ovule contains a megasporangium surrounded and protected by one or two integuments. The megasporangium retains a single megaspore, which produces the female gametophyte (Herr, 1995). After double fertilization, the embryo develops from the zygote, and fusion between the two polar nuclei and a sperm cell nucleus leads to formation of the endosperm. During seed development, the integument(s) undergoes morphological changes and becomes the seed coat.

Many studies have focused on the molecular control of either ovule development or seed formation; however, it has been difficult to determine a relationship between the two developmental programs. A number of Arabidopsis mutants displaying aberrant ovule development have been described. The short integuments (sin1) mutant has short integuments that fail to cover the nucellus completely (Robinson-Beers et al., 1992; reviewed in Gasser and Robinson-Beers, 1993). Superman (sup) ovules lack asymmetric growth of the outer integument, resulting in elongated ovules of reduced fertility and suggesting a role for SUP in cellular proliferation (Gaiser et al., 1995; Sakai et al., 1995). In bell, the best-characterized ovule mutant, the outer integument is often replaced by a carpel-like structure (Robinson-Beers et al., 1992; Modrusan et al., 1994; Ray et al., 1994; Reiser et al., 1995); in the inner-no-outer (ino) mutant, the outer integument initiates but fails to develop further (Gaiser et al., 1995). Recently, the aintegumenta (ant) mutant has been described. For ant mutants, the development of the integuments is not initiated, and megasporogenesis is blocked at the tetrad stage (Elliott et al., 1996; Klucher et al., 1996).

On the other hand, several Arabidopsis seed coat mutants have been described. transparent testa (tt) lacks pigmentation of the seed coat because of mutations of genes involved in the flavonoid biosynthetic pathway (Koornneef, 1990); transparent testa glabra (ttg), glabrous2 (g12), and aberrant

†Current address: Dipartimento di Genetica e Biologia dei Microorganismi, via Celoria 26, Milan, Italy.
‡To whom correspondence should be addressed. E-mail g.c.angenent @cpro.dlo.nl; fax 31-317-418094.
testa shape (ats) are three other mutants in which seed coat development is affected (Koornneef, 1981; Léon-Kloosterziel et al., 1994; Meyerowitz and Somerville, 1994). In addition, in the homeotic apetala2 (ap2) mutant, the seed coat is also affected without changes in endosperm and embryo development (Jofuku et al., 1994). Thus, to date, evidence that endosperm and embryo development in Arabidopsis depends on normal seed coat development is lacking.

One of the best-described class of plant regulatory genes is the MADS box gene family. The MADS box transcription factors play essential roles in determining meristem and floral organ identity, and some of them are also expressed during ovule and seed development. Recently, two petunia MADS box genes, Floral Binding Protein7 (FBP7) and FBP11, have been identified, and their role in ovule development has been investigated (Angenent et al., 1995; Colombo et al., 1995). Overexpression of these genes resulted in ectopic ovule formation on the two outer floral whorl organs, indicating their involvement in ovule initiation and ovule identity specification. This observation has led to the extension of the ABC model (reviewed in Coen and Meyerowitz, 1991) with a D function. The D function is required to specify the identity of the fifth floral organ type, the ovule (Colombo et al., 1995). Several other MADS box genes are expressed during ovule and embryo development. For example, the Arabidopsis MADS box genes AGAMOUS-like2 (AGL2), AGL11, and AGL13 are expressed in ovules and, at a reduced level, during seed formation (Flanagan and Ma, 1994; Rounsley et al., 1995), and AGL15 is specifically expressed in the embryo (Heck et al., 1995); however, their functions have not been determined yet.

In conclusion, ovule and seed development have often been studied separately, and little is known about the development of the maternally derived tissues of the seed and the genes that control this process. In this report, we analyze the expression of the petunia MADS box genes FBP7 and FBP11 after fertilization and their role in seed development. The activity of the FBP7 promoter was monitored by using the β-glucuronidase (GUS), luciferase (LUC), and BARNASE reporter gene fusions driven by the FBP7 promoter. To analyze the role of FBP7 and FBP11 at later stages of ovule ontogeny, we compared seed development in wild-type petunia plants with that in transfectants in which both MADS box genes were downregulated by cosuppression. This study provides new insights into the developmental interactions of the embryo, endosperm, and seed coat in a dicotyledonous plant species.

RESULTS

Expression of FBP7 and FBP11 Genes during Flower Development

It has been previously reported that the petunia MADS box genes FBP7 and FBP11 are specifically expressed in ovules (Angenent et al., 1995). Transcripts of these genes are first detectable in the central meristem of the petunia flower bud, which is the progenitor tissue of the placenta and ovules. Later, these transcripts are restricted to the ovules, predominantly in the endothelium, which is the innermost cell layer of the integument (Angenent et al., 1995). To study the expression of these MADS box genes in developing seeds, RNA gel blot analyses were performed using RNA isolated from ovaries at various stages after pollination. Figure 1A shows an RNA gel blot hybridized with probes specific for FBP7, FBP11, or pMADS3 (Tsuchimoto et al., 1993). The expression levels of both FBP7 and FBP11 increased immediately after pollination (2 days after pollination [DAP]) and declined in mature seeds. In the same experiment, we followed the expression of pMADS3 during the same stages. pMADS3 is the putative petunia class C homeotic gene homologous to AG (Yanofsky et al., 1990) and PLENA (Bradley et al., 1983), which is also expressed in ovules (Tsuchimoto et al., 1993; M. Kater, L. Colombo, and G.C. Angenent, manuscript in preparation). In contrast to FBP7 and FBP11, pMADS3 expression decreased after pollination (Figure 1A).

The accumulation of FBP7 and FBP11 proteins in the developing seeds was also monitored by protein gel blot analysis (Figure 1B). Polyclonal antibodies were raised against the C-terminal part of FBP11, but they also recognized FBP7 because of the high levels of similarity between these
two polypeptide sequences. This cross-reactivity was demonstrated by protein gel blots of extracts from sepals belonging to transgenic plants overexpressing either the FBP7 or the FBP11 gene (data not shown). Greater amounts of FBP7 and FBP11 proteins were detectable in nuclear extracts from ovules after pollination compared with extracts from ovules before the fertilization events. The smaller protein band in the protein gel blot shown in Figure 1B is always accompanied by the larger FBP7/FBP11 band and probably corresponds to a degradation product.

To determine the distribution of FBP7 mRNA in ovules before and after fertilization, we performed in situ hybridizations, using a 3' terminal antisense transcript of FBP7 as probe. Figures 2A and 2B show the expression of FBP7 in ovules at two stages of development. This expression pattern is very similar to that of FBP11, as has been reported previously (Angenent et al., 1995). In ovules 3 and 5 DAP (Figures 2C and 2D), strong hybridization signals were observed in the tissues that form the seed coat, whereas very

![Figure 2](image-url)

**Figure 2.** Expression of the FBP7 Gene during Ovule Development before and after Fertilization.

Longitudinal sections were hybridized with the digoxigenin-labeled antisense FBP7 transcript.

(A) Ovary containing ovules at stage 12 (Angenent et al., 1995), in which the integument covers the nucellus completely.

(B) Mature unfertilized ovules.

(C) Fertilized ovules 3 DAP.

(D) Fertilized ovules 5 DAP.

cw, carpel wall; o, ovule; pi, placenta; sc, seed coat. Bars in (A) and (B) = 1.0 mm. The magnifications are the same for (A) and (C) and for (B) and (D).
Figure 4. Activity of the FBP7 Promoter Using the Reporter Genes GUS, LUC, and BARNASE.
low or no expression was found in other ovary tissues. Similar expression patterns were observed by using an FBP11-specific antisense probe (data not shown). These expression data clearly show that the petunia MADS box genes FBP7 and FBP11 are specifically expressed in ovules and that their expression persists and even increases significantly during seed development. This suggests that FBP7 and FBP11 are not only required for ovule development but may also be involved in postfertilization processes.

**FBP7 Promoter Activity during Ovule Ontogeny**

To study the regulation of FBP7 expression during seed development in more detail, we have isolated a 0.6-kb FBP7 promoter fragment by inverse polymerase chain reaction. Two chimeric gene constructs were made and consisted of the FBP7 promoter fragment fused to either the GUS or LUC reporter gene. These chimeric constructs, schematically shown in Figure 3, were introduced into petunia line W115 by using Agrobacterium-mediated transformation (Horsch et al., 1985), and the resulting plants were tested fluorometrically for GUS activity and luminometrically for LUC activity.

All GUS-positive plants (four of 27 transformants, namely, T51002, T51009, T51013, and T51027) showed a high level of GUS activity in the ovules. GUS activity in ovules of T51013 before fertilization is shown in Figure 4A, with the activity increasing 4 DAP (compare Figures 4A and 4B). GUS activity was mainly present in the developing seed coat, excluding the outer cell layer, and in the epidermis of the placenta (Figure 4C). Although GUS is a suitable reporter gene to determine tissue specificity, LUC is a far more precise reporter protein to study the temporal and relative activity of the FBP7 promoter.

To monitor LUC expression driven by the FBP7 promoter, we selected one (T59042) of 28 transformants in which promoter activity was high enough for a clear luminescence signal after 30 min of measurement. To avoid accumulation effects, flower buds were treated daily with substrate before measurement, enabling the detection of de novo transcription activity. The expression at various stages of ovary development after pollination is shown in Figure 4D. Photon production due to the activity of the LUC gene driven by the FBP7 promoter was detectable in mature ovules but showed an increase after pollination, reaching a maximum at ~7 DAP. Activity declined to almost zero at ~14 DAP (Figure 4D). The increase in activity a few days after pollination was specific for the FBP7 promoter, because it was not seen in transgenic control plants in which LUC was driven by the cauliflower mosaic virus 35S promoter (data not shown). However, the decrease in LUC activity ~14 DAP is a general phenomenon due to the initial desiccation of the seed and was also seen in the control plants. Taken together, our data show that FBP7 promoter activity coincides exactly with the pattern of FBP7 expression during ovule and seed development, as was determined by RNA blot analysis and in situ hybridization.

**BARNAASE Gene Expression Controlled by the FBP7 Promoter**

The activity of the FBP7 promoter was also monitored by fusing it to the bacterial BARNAASE gene (Hartley, 1988), as shown in Figure 3. The effect of expression of this cytotoxic gene was analyzed in 26 independently generated primary transformants, namely, T52001 to T52026. The severity of the defects varied considerably among transformants. Two major classes of transformants were identified. In the first class of transformants (eight plants), the flower buds did not reach maturity and the pistil was completely missing (compare Figures 4E and 4F). The second class of transformants (five plants) had normal flowers with reduced pistils in which no ovules developed (compare Figures 4G and 4H).

Figure 4. (continued).

(A) Cross-section through a mature ovary of the transgenic plant T51013 containing the FBP7 promoter-GUS construct. Blue staining indicates GUS activity.

(B) GUS activity in a cross-section through a T51013 ovary 7 DAP.

(C) GUS activity in a longitudinal section through a T51013 ovary 7 DAP. The section was viewed by dark-field microscopy, with the signal color being red.

(D) LUC activity driven by the FBP7 promoter in mature T59042 ovaries (0 DAP) and ovaries 4, 7, 9, and 14 DAP. The carpel walls were removed before spraying. The color bar, from gray to red, indicates an increasing intensity of LUC activity.

(E) Wild-type flower bud of line W115. Sepals, petals, and anthers are partly removed.

(F) Detailed view of a small bud of transgenic plant T52012 expressing the BARNAASE gene. The carpel is completely missing, and the development of flower organs is blocked at a young stage. The arrow indicates ablated cells (brown) in the receptacle at a position where normally the pistil develops.

(G) Wild-type ovary with removed ovary wall.

(H) Ovary of a T52006 flower. No ovules were observed after removing the ovary wall.

ow, carpel wall; o, ovules; p, placenta; sc, seed coat.
Figure 5. Microscopic Analysis of Seed Development from Wild-Type and T27017 Plants.
other 13 plants had an intermediate phenotype, and they were not analyzed further.

One plant (T52006) belonging to the second class of transformants was used to carefully monitor FBP7 promoter activity in seed tissue of gametophytic origin. Wild-type plants were pollinated with pollen from the T52006 transformant, and the developing seeds were examined from 1 DAP until they were mature. No aberrations were found during the development of the seeds, and all seeds examined germinated normally. However, approximately half of the progeny plants exhibited the same aberrant phenotype, as did the primary transformant T52006, because of BARNASE activity. Transformant T52006 contains three T-DNA insertions that segregate as one locus. These BARNASE transgenes were present only in the progeny plants with defective pistils, as was confirmed by DNA gel blot analysis (data not shown). The most likely explanation for the transmission of the highly effective killer gene BARNASE to the progeny is that the activity of the FBP7 promoter in the developing seed is restricted to the seed coat and that it is not active in the gametophytically derived tissues, the embryo and endosperm. Therefore, a putative role for FBP7 during seed development might be restricted to the formation of the seed coat.

**FBP7** and FBP11 Are Required for Proper Seed Development

During the double fertilization process, one male gamete fertilizes the egg cell, giving rise to the zygote, whereas the second male gamete fuses with the diploid central cell, forming the triploid primary endosperm nucleus. The development of a mature embryo in petunia line W115 requires ~4 weeks, with temperature and day length influencing the actual time required for embryo maturation (Sink, 1984). The seed coat, which is derived from the single integument of the petunia ovule, is thought to contribute to seed formation by distributing essential nutrients to the developing endosperm and embryo (Murray, 1984, 1987).

To investigate the function of **FBP7** and FBP11 during seed formation, we compared the development of wild-type petunia seeds and seeds derived from transgenic plants in which **FBP7** and FBP11 were simultaneously downregulated by cosuppression (Angenent et al., 1995). Three transgenic plants (T27017, T27035, and T27040) were obtained showing the same cosuppression phenotype as was described previously (Angenent et al., 1995). One of these transgenic plants, T27017, was used for further investigation. In this transgenic plant, the expression of both **FBP7** and FBP11 genes was reduced >10-fold, and a similar reduction was observed after fertilization (data not shown). A few ovules were transformed into carpelloid structures, whereas the majority of the ovules were morphologically normal and developed into seeds upon fertilization.

In contrast to the round-shaped seeds from a wild-type plant (Figure 5G), the seeds obtained by self-pollination of the transgenic plants (T27017, T27035, and T27040) were shrunken, as shown in Figure 5H. To investigate the inheritance of seed phenotype, we performed several crosses. All seeds from the self-pollination of the T27017 plant and the seeds derived after pollination with wild-type pollen exhibited the shrunken phenotype. In contrast, seeds from backcrosses using pollen of transgenic origin to fertilize wild-type ovules were all phenotypically normal. The cross between wild-type and T27017 plants, in both directions, gave rise to progeny in which 19 of 35 plants for the cross W115 × T27017 and 16 of 30 plants for the cross T27017 × W115 had wild-type phenotypes. These results indicate that the shrunken seed phenotype is not lethal and is of maternal origin.

Light microscopic analysis was performed to investigate the internal anatomy of mature seeds from both the wild type and mutants. During development of wild-type and T27017 seed, the cellularization of the endosperm was comparable in both types until 9 DAP (Figures 5A and 5B). However, between 13 and 21 DAP, T27017 seeds showed many
Figure 6. Seed Coat and Endosperm Development of the Wild Type and the T27017 Mutant of Petunia.
different forms of endosperm degeneration within one ovary. In the less severe forms, endosperm degeneration occurred at later stages of development and became apparent near maturity (compare Figures 5C and 5D). The mature seeds of the wild type showed intact endosperm and a fully developed embryo (Figures 5E and 5I), whereas in mature T27017 seeds, the endosperm was partly or totally degenerated (Figure 5F), and dissected embryos varied from globular and heart shaped to fully developed (Figure 5J).

To study the aberrant seed phenotype in more detail, we analyzed sections of seeds at different developmental stages. This microscopic analysis is shown in Figure 6. The petunia integument, which differentiates into the seed coat, consists of an outer epidermis, an inner epidermis called the endothelium, and approximately six layers of parenchyma cells (Figure 6A). In wild-type seed development (Figures 6A to 6E), the outer epidermis cells enlarged during development, stored large quantities of a substance that stained with toluidine blue (asterisk in Figure 6B), and formed a thick tangential cell wall against the parenchyma cells. The endothelium cells were papillar shaped at 5 DAP (Figure 6A) and were flattened after 9 DAP (Figure 6B). Subsequently, they also stored a substance that stained with toluidine blue, as shown in Figures 6C and 6D. After 18 DAP, the parenchyma cells and endothelium degenerated, resulting in one layer of parenchyma cells between the outer epidermis and the endosperm. The endosperm and embryos at 21 DAP were rich in cytoplasm and storage products (Figure 6E).

The development of the T27017 seed coat (Figures 6F to 6J) was similar to the wild type at 5 and 9 DAP (compare Figures 6A with 6F, and 6B with 6G). At 13 DAP, differences in the wild-type seed coat were observed (compare Figures 6C and 6H): in some cases, a part of the endothelium of T27017 was degenerated (indicated with arrows), whereas adjacent endothelium cells were intact (Figure 6H). The endosperm cells bordering the degenerating endothelium also exhibited degeneration of cytoplasm (Figure 6H), whereas the endosperm cells bordering the intact endothelium showed less intense staining and were indistinguishable from cells found in wild-type endosperm. The endothelium cells were completely degenerated after 18 DAP (Figures 6I and 6J). The endosperm cells at 21 DAP were highly vacuolated and partly or completely degenerated, as shown in Figure 6J. The embryo developed more slowly in the mutant (Figure 6J) than in wild-type seeds (Figure 6E), and embryos arrested in their development were observed. Nevertheless, many T27017 seeds develop a viable embryo despite the aberration of the seed coat and endosperm.

**DISCUSSION**

Seed development is an important process during the life cycle of a plant. Here, we describe the role of two petunia MADS box genes, FBP7 and FBP11, in seed formation. Both FBP7 and FBP11 are essential for the determination of petunia ovule identity and development (Angenent et al., 1995; Colombo et al., 1995). By analyzing seed development in plants in which FBP7 and FBP11 are simultaneously cosuppressed (Angenent et al., 1995), we have shown that the expression of both genes is also required for normal seed development. In this report, we demonstrate the role of a MADS box transcription factor in the development of the seed coat. In addition, new information is provided about the interrelationships of the three types of tissue that compose...
Expression Pattern of FBP7 and FBP11 in Developing Seeds

In wild-type petunia plants 2 DAP, when all of the ovules have been fertilized, the expression of both FBP7 and FBP11 is increased. High expression levels are maintained throughout the maturation of the seeds and decrease at later stages when the seeds start to desiccate. The expression of pMADS3, another MADS box gene expressed in ovules, decreases after pollination, indicating that the induction of FBP7 and FBP11 expression is specific for these two genes. In situ hybridization experiments using FBP7 and FBP11 antisense RNAs as probes confirmed RNA gel blot data and showed that their expression is very high in seed tissue derived from the ovule integument.

The putative Arabidopsis ortholog of FBP11 and FBP7, AGL11, has been isolated and characterized (Rounsley et al., 1995). The expression pattern of this gene is very similar to the those of FBP11 and FBP7. AGL11 starts to be expressed in the placental tissue before the ovules arise; later in development, its expression is localized in the ovules and placental tissue. In ovules, its expression is mainly restricted to the two integuments; after fertilization, AGL11 is predominantly expressed in the chalazal region of the ovule (Rounsley et al., 1995). However, in contrast to FBP7 and FBP11, whose expression was greatly increased after pollination, AGL11 expression is not detectable by RNA gel blot analysis after fertilization. This suggests either that AGL11 is not the true ortholog of FBP11 and FBP7 or that the function of this gene is restricted to ovule development, and it is not required for Arabidopsis seed formation.

FBP7 is Expressed Exclusively in Maternal Tissue of the Seed

We isolated the FBP7 promoter to investigate FBP7 expression in detail by using reporter and cell ablation genes. Each reporter gene that was used provided us with specific information about FBP7 gene expression in the developing seed. Because no active LUC accumulates when the plants are regularly sprayed with luciferin (Millar et al., 1992; Wood, 1995), LUC activity reflects de novo reporter gene expression. On the other hand, the GUS reporter gene was used as a tool to study tissue specificity in the seed, and high activity was observed in a major part of the seed coat.

To monitor traces of FBP7 promoter activity in embryo and endosperm tissues, we performed cell ablation, using the highly sensitive bacterial RNase BARNASE. Transgenic plants specifically expressing BARNASE in the ovules were used to study the activity of the FBP7 promoter during seed development. Backcrosses with wild-type plants, using pollen carrying the FBP7 promoter–BARNASE construct, revealed that the BARNASE phenotype can be transferred through the pollen to the progeny. The most likely explanation for this observation is that the promoter is active in maternally originated tissue of the developing seed and not in seed tissues derived from the double-fertilization process. However, it cannot be excluded that the transgene, when contributed by the male, may be silenced in endosperm and embryo.

Role of FBP7 and FBP11 in Seed Development

The induction of FBP7 and FBP11 expression after fertilization supports the idea that these genes are involved in seed development. To study this proposed function, we followed the formation of seeds in the wild type and in transgenic plants in which the expression of both FBP7 and FBP11 was dramatically reduced by cosuppression. Microscopic analysis has shown that in mature seeds from these transgenic plants, the development of the endosperm is negatively affected, resulting in a shrunken seed phenotype. Genetic analysis showed that the aberrant seed phenotype is maternally inherited. Analyses of early and intermediate developmental stages by light microscopy revealed that up to 9 DAP, the development of T27017 seeds is similar to wild-type seed development. However, between 9 and 13 DAP, the inner epidermis of the seed coat, the endothelium, starts to degenerate. At 18 DAP, the endothelium is completely degenerated, and this directly disturbs the development of the endosperm, probably because of a block in transport of nutrients through the seed coat to the developing endosperm and embryo. This leads to the formation of mature seeds with or without a small amount of endosperm. Genetic analysis showed that the aberrant seed phenotype is maternally inherited, indicating that the defects in endosperm development are indirect. The phenotype of the cosuppression mutant suggests that FBP7 and FBP11 MADS box genes play a role in the development of the seed coat.

The interaction of endosperm, embryo, and maternal tissues remains one of the most complex and unresolved aspects of seed development. It has been suggested that maternal and endosperm tissues may regulate each other’s development (reviewed in Lopes and Larkins, 1993). Cell proliferation in the endosperm and protein and starch biosynthesis largely depend on the nutrient flow from the maternal tissues. Defects in this part of the seed coat that facilitates this transport of nutrient, which is believed to be the endothelium, may have negative effects on the development of the endosperm, which ultimately degenerates. In contrast, several Arabidopsis mutants have been described in which embryo and endosperm develop normally despite aberrations in seed coat development (Jofuku et al., 1994; Léon-Kloosterziel et al., 1994). Apparently, the defects in seed coat development occur late during seed development or they do not affect nutrient flow.
In this report, we demonstrate the interaction between the seed coat and endosperm. Despite the degeneration of the endosperm, normal embryo development was observed, suggesting that at later stages of petunia seed development, the endosperm is not required for proper embryo formation. That this may be a general phenomenon for all dicot seeds is supported by the fact that in contrast to monocotyledonous seeds, mature dicot seeds contain only a small amount of endosperm.

METHODS

Plant Material

The Petunia hybrida variety W115 and the transgenic plants were grown under normal greenhouse conditions.

DNA and RNA Gel Blot Analyses

Plant DNA was isolated from petunia leaves, according to Koes et al. (1990), and total RNA was isolated from ovaries, according to Vervoord et al. (1989). Ten micrograms of DNA was digested with EcoRI or 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) before electrophoresis. Equal loading of RNA in the gel slots was verified by ethidium bromide staining of the gel. Floral Binding Protein7 (FBP7) (0.6 kb), FBP11 (0.79 kb), and pMADS3 (0.6 kb) 3′ terminal fragments and the FBP7 (0.38 kb) 5′ terminal fragment were labeled by random oligonucleotide priming (Feinberg and Vogelstein, 1984). Blots were hybridized, washed, and stained with a solution of 1% toluidine blue in distilled water.

In Situ RNA Hybridizations

Floral buds were fixed and embedded in paraffin, and 10-μm sections were prepared as described by Cañas et al. (1994). Digoxigenin-labeled RNA probes were synthesized by in vitro transcription using the pSPT18/19 vectors (Boehringer Mannheim). For the synthesis of antisense RNA, we introduced 3′ terminal cDNA fragments of FBP7 (0.6 kb) into pSPT18 or pSPT19 by using the internal restriction site HindIII. Transcripts were partially hydrolyzed by incubation at 60°C in 0.1 M Na₂CO₃-NaHCO₃ buffer, pH 10.2, for 45 min. Hybridization and immunological detection were performed as described by Angenent et al. (1992).

Microscopy

The material was fixed in 5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The material was rinsed three times for 10 min in 0.1 M sodium phosphate buffer, pH 7.2, and then dehydrated using a series of alcohol solutions (30, 50, 70, 90, and 100%) for 30 min each. The material was embedded in hydroxyethyl methacrylate Technovit 7100, according to the manufacturer's protocol (Kulzer Histo-tec, Wehrheim, Germany). After embedding, the material was placed in a mold, and 1 mL of hardener II (Kulzer Histo-tec) was added per 15 mL of Technovit, resulting in a polymerization reaction. The Technovit blocks were sectioned by microtome. Sections were stained with a solution of 1% toluidine blue in distilled water.

For cryoscanning, electron microscopy samples were mounted on a stub, frozen in liquid nitrogen, coated, and observed as described in Angenent et al. (1995). Ovules 7, 14, and 21 days after pollination (DAP) were histochemically stained for β-glucuronidase (GUS) activity, according to Koes et al. (1990), before microscopic sections were made.

Bacterial Expression of FBP7 and FBP11, Immunization, and Immunoblot Analysis

A 3′ FBP11 terminal cDNA fragment (0.65 kb) was cloned in the bacterial expression vector pET11d (Rosemberg et al., 1987) by using Nool (5′) and BamHI (3′) sites. Both sites were generated by polymerase chain reaction (PCR), using a 5′ primer (ISOGEn; Bioscience BV, Maarssen, The Netherlands) (5′-CGCCATGGCAATAGGGCTCTTG- GTGGTGAAAG–3′; 361 bp downstream of the ATG codon) and 3′ primer (5′-GAGCAGATTCCTTCTTCGCGGCG–3′; 40 bp downstream of the stop codon), respectively. The underlined nucleotides correspond to the cDNA. The N-terminal 106 amino acid residues were removed to avoid cross-reactions with other MADS box proteins. This construct was introduced into BI21, a strain of Escherichia coli containing an integrated copy of the isopropyl β-D-thiogalactopyranoside (IPTG) gene (Rosemberg et al., 1987). The cells were grown, harvested, and sonicated, according to Angenent et al. (1992). After centrifugation, the pellet was resuspended in loading buffer, according to Laemmli (1970). A New Zealand rabbit was immunized with 100 μg of FBP11 protein purified from an SDS-polyacrylamide gel. Booster injections with the same amount of protein were given twice with an intervening period of 28 days, and the serum was collected after an additional 10 days. Mature ovules and ovules 1, 2, 3, 4, 7, and 21 DAP were used for nuclear protein extraction, according to the method described by Angenent et al. (1992). Protein gel was made by using 100 μg of nuclear proteins for each sample. The protein detection was done according to Angenent et al. (1992).

Isolation of the FBP7 Promoter Fragment by Inverse PCR

Three micrograms of genomic DNA (line W115) was digested with HindIII in a final volume of 400 μL. The restriction reaction was performed for 10 hr and followed by a phenol-chloroform extraction procedure. After DNA precipitation, a ligation reaction was performed using 10 units of T4 DNA ligase in 500 μL final volume at 16°C for 16 hr. After precipitation, ~0.5 μg of the DNA was used in the PCR. In the first PCR (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C for 30 cycles), a primer (5′-GTGCCATGGGAGAGAGATAAGAG–3′) containing the ATG codon (underlined) and a primer containing the ATG codon (underlined) but with the opposite orientation (5′-GAAGATTCCTTCCATGGCACATATAATATTCTGATTAG–3′) were used. In the second PCR (same conditions as were given for the first one), the first primer was substituted by a nested primer (5′-CGGGATC- CGGTCTGCGAAGAGAAAATG–3′; 58 bp downstream of the ATG). An amplified product of 620 bp was obtained. The promoter and fragment was cloned into pEMBL19 and sequenced. The GenBank and EMBL accession number of this promoter fragment is U90137.
Construction of Binary Vectors and Plant Transformation

The fbp7 promoter was cloned into the binary vector pBin19. The GUS gene, the LUCIFERASE (LUC) gene, or the BARNASE-BARSTAR bacterial operon construct (Hartley, 1998) was cloned downstream of the promoter. The recombinant pBin19 vectors were transferred via Agrobacterium tumefaciens (LBA4404) to petunia variety W115, using the standard leaf disc transformation method (Horsch et al., 1985). Regeneration of transformants was done as described by Van Tunen et al. (1989).

LUC Activity Measurements

Flowers of plants carrying the FBP7-LUC construct were sprayed twice a day with a solution of luciferin (1 mM), starting 2 days before measurement. At harvest, the sepals, petals, and stamens as well as the carpel walls were carefully removed to expose the developing seeds. Imaging of photon production was done for 30 min with a two-dimensional luminometer (Hamamatsu, Hamamatsu, Japan), using the Argus 50 software (Hamamatsu).

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