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The phenotype of *Arabidopsis* ovule mutants mimics the morphology of primitive seed plants

Sung Ok Park, Soon Hwang and Bernard A. Hauser*

Department of Botany, 220 Bartram Hall, University of Florida, Gainesville, FL 32611-8526, USA

In seed plants, the ovule is the female reproductive structure, which surrounds and nourishes the gametophyte and embryo. This investigation describes the *PRETTY FEW SEEDS2* (*PFS2*) locus, which regulates ovule patterning. The *pfs2* mutant exhibited developmental defects in the maternal integuments and gametophyte. This mutation was inherited as a maternal trait, indicating that gametophyte defects resulted from ovule patterning aberrations. Specifically, the boundary between the chalaza and the nucellus, two regions of the ovule primordia, shifted towards the distal end of *pfs2* ovule primordia. Results indicated that the *PFS2* locus could: (i) be involved in the development of either the nucellus or the chalaza; or (ii) establish a boundary between these two regions. Examination of genetic interactions of the *pfs2* mutation with other well-characterized ovule loci indicates that this locus affects integument morphogenesis. Interestingly, the *pfs2 inner no outer* and *pfs2 strubbelig* double mutants had inner integuments that appeared similar to their ancestral precursor. The fossil record indicates that the inner integument evolved by fusion of sterilized sporangia or branches around a central megasporangium. The question of whether the structures observed in these double mutants are homologous or merely analogous to the ancestral precursors of the inner integument is discussed.

Keywords: *Arabidopsis thaliana*; integument evolution; *pretty few seeds2* mutant; plant reproduction; telome

1. INTRODUCTION

One of the most important adaptations made by land plants was the evolution of ovules, which are the direct precursors of seeds. In aqueous environments, plant spores simply travel through water. When plants colonized land, they needed to develop an efficient method of fertilization across geographical distances that did not require water. Land plants evolved prepollen that travelled through the air until they encountered a female spore or megasporangium (Raven *et al.* 1999). Female reproductive structures or megasporangia evolved into ovules with pollination droplets, pollen chambers and/or hairs to capture the male spores or prepollen (Niklas 1997). Prepollen, which had not yet developed the capacity to produce pollen tubes, probably travelled from the pollen chamber to the ovule via motile antherozoids (Poort *et al.* 1996). Subsequently, more elaborate methods of pollen transmission evolved, including pollen tubes, animal pollinators, floral structures to attract these pollinators and the enclosure of ovules within a carpel. In spite of the greater geographical distance between individuals, the evolution of ovules and pollen in seed plants increased the efficiency of sexual mating in these land plants. As a consequence of these adaptations, seed plants became the dominant land plants.

An important process in ovule evolution was the evolution of a mechanism to capture wind-borne pollen. In a number of extinct taxa, a series of lobed or filamentous processes partially surrounded the megasporangia (Andrews 1963). It has been hypothesized that these lobes deflected pollen towards the megasporangia (Niklas 1981). There is a direct relationship between the degree of organization of these lobes and the sophistication of the pollen-receiving mechanism (Andrews 1963). Other pollen-trapping mechanisms included pollination droplets, hairs or other processes that facilitated pollen adhesion.

Based on fossil evidence of reproductive structures in extinct plants, palaeobotanists proposed that integuments evolved from the fusion of lobed structures surrounding a sporangium (Stewart 1983; Herr 1995; Kenrick & Crane 1997). Two theories, the telome theory and the synagium theory, attempt to account for inner integument evolution. The telome theory (Zimmerman 1952) postulates that peripheral sterile lateral branches fused together to enclose a central megasporangium, thereby forming an integument. Alternatively, the synagium theory (Benson 1904) postulated that the angiosperm inner integument arose exclusively from fusion of modified sporangia around a central fertile sporangium. Although the exact origin of telomes is still debated, fossil evidence indicates that telomes fused together to form an integument.

Although little is known about the genes involved in the evolution of ovules, the development of wild-type *Arabidopsis* ovules has been well characterized (Robinson-Beers *et al.* 1992; Modrusan *et al.* 1994; Schneitz *et al.* 1995). In this model angiosperm species, genetic and molecular approaches were used to delineate the developmental pathway for ovules. These studies have made steady progress in identifying and predicting additional loci that regulate steps of ovule development (Gasser *et al.* 1998; Grossniklaus & Schneitz 1998). Herein the *pretty few seeds2* (*pfs2*) mutation is described. This mutation causes a reduction in plant fertility, which stems from defects in the patterning of ovule primordia. In addition, the *pfs2* mutant displays dramatic interactions with other ovule mutations.

* Author for correspondence (bahauser@botany.ufl.edu).

2. MATERIAL AND METHODS

(a) *Plant growth*

Plants were grown on autoclaved soil (Fafard Mix No. 2, Conrad Fafard Inc., Agawam, MA, USA) at a fluence rate of *ca.* 100 μEinstein m^{-2} s^{-1}. Plants were T-DNA mutagenized and the M2 progeny were screened for defects in ovule morphology. The mutant isolates were backcrossed to wild-type Landsberg *erecta* (L*er*) three times before phenotypic and genetic analyses were undertaken. Two *pfs2* mutant alleles were found.

(b) *Double-mutant analysis*

The *pfs2-1* mutant was crossed with the following ovule mutants: *inner no outer-1* (*ino-1*), *nozzle-2* (*nzz-2*) and *strubbelig-1* (*sub-1*) (Schiefthaler *et al.* 1999; Villanueva *et al.* 1999; Chevalier & Schneitz 2000). The *nzz* mutant was independently described as *sporocyteless* (Yang *et al.* 1999), but for simplicity we will use *nzz* in this paper. A stereomicroscope was used to observe ovule morphology and phenotypes in segregating double-mutant populations.

(c) *Microscopy*

Pistils were prepared for scanning electron microscopy (SEM) as described by Robinson-Beers *et al.* (1992). Specimens were examined and digital images were captured with a Hitachi S-4000 FE-SEM (Tokyo, Japan). To examine ovules using Normarsky optics, ovules were fixed, cleared and visualized as described by Herr (1971). To examine sectioned material, pistils were fixed in 1.5% glutaraldehyde, 0.1% Tween 20 and 25 mM of cacodylate buffered to a pH of 7.0, embedded in Spurr's resin, sectioned and stained with thionine and acridine orange (Spurr 1969; Paul 1980).

(d) *Mapping*

Ovule mutants, in the L*er* genetic background, were crossed to wild-type Columbia-3 plants. In F2 mutants from this cross, the genotypes at various loci were determined using PCR-based genetic markers (Konieczny & Ausubel 1993). For genetic markers that were linked to the *PFS2* locus, genetic distances were determined using MapMaker v. 2.0 (Lander *et al.* 1987).

3. RESULTS

(a) *Reduced fecundity in* pretty few seeds2 *mutants*

Two *pfs2* alleles were identified. Populations segregating for alleles at this locus had 384 wild-type plants and 126 *pfs2* mutants, which closely correlated with the 3 : 1 segregation ratio expected for a single-locus recessive mutation ($\chi^2 = 0.024$, $p = 0.88$). When compared with wild-type ovules, *pfs2-1* and *pfs2-2* mutants exhibited reduced fertility. In 30 randomly selected pistils, the numbers of viable ovules in wild-type L*er* and *pfs2* mutants were counted. Pistils from the *pfs2-1* and *pfs2-2* mutants developed 2.0 ± 1.2 and 27 ± 3.3 seeds per fruit, respectively. When grown under the same environmental conditions, the wild-type L*er* ecotype produced an average of 47.2 ± 4.8 seeds per fruit. Both *pfs2* alleles yielded significantly fewer seeds than did the wild-type plants ($p < 0.01$). Thus, the majority of ovules in *pfs2* mutants aborted before forming seeds.

(b) *Anatomy of wild-type and* pretty few seeds2 *mutant ovules*

In wild-type plants, ovule primordia differentiated into three developmental zones: the funiculus, chalaza and nucellus (figure 1*a*). Within the nucellus, the megaspore mother cell undergoes meiosis. A single meiotic product divided and differentiated into a gametophyte. The integuments arose from the chalaza and ultimately enveloped the nucellus, leaving only a small opening, the micropyle, where the pollen tube can enter the ovule (figure 1*d*).

In most *pfs2* ovules, the embryo sac either aborted during development (figure 1*e*), or was anatomically aberrant. If present, the embryo sac in *pfs2* ovules was usually reduced in size and had fewer cells than the wild-type embryo sac (data not shown). The reduction in embryo-sac size probably arose from early defects during differentiation of the megaspore mother cell. In wild-type ovules, the megaspore mother cell was quite large, occupying most of the space in the nucellus (shaded region in figure 1*b*). In the nucellus of *pfs2-1* ovules, however, the megaspore mother cell was reduced in size (shaded region in figure 1*c*) and additional cells were found in this region (arrowheads in figure 1*c*). In addition, the boundary between the chalaza and the nucellus was often poorly defined in these mutants (figure 1*c*). The *pfs2-2* allele led to similar problems with ovule patterning, but at a reduced frequency (data not shown). As a consequence of these changes in early ovule patterning, subsequent formation of the gametophyte was disrupted in most ovules.

(c) *The* PRETTY FEW SEEDS2 *locus*

Because there were defects in both the sporophyte and gametophyte of *pfs2* ovules, the inheritance of this locus was closely examined. It was important to learn whether this locus was inherited as a maternal (sporophyte) mutation or a gametophyte mutation. Consequently, the ovules produced by *pfs2* heterozygotes were examined for embryo-sac defects. In these ovules, the maternal tissue was heterozygous (phenotypically wild-type), while the genotype of the haploid gametophyte or embryo sac segregated 1 : 1 for the *pfs2* and wild-type alleles. The anatomy of each embryo sac ($n = 25$) was indistinguishable from the wild-type. Because half of these gametophytes would carry the mutant *pfs2* allele, we can exclude the hypothesis that *pfs2* is inherited as a gametophyte mutation ($\chi^2 = 25.0$, $p = 5.7 \times 10^{-7}$). Rather, the data indicate that *pfs2* is a sporophyte mutant with maternal effects on gametophyte development.

Genetic interactions of *pfs2* with characterized ovule mutants were evaluated to determine whether *pfs2* was allelic to an existing locus. The following mutants complemented *pfs2-1*: *lalle*, *sub*, *mollig*, *blassig*, *aintegumenta*, *nzz*, *ino* and *short integuments1* (Robinson-Beers *et al.* 1992; Elliott *et al.* 1996; Klucher *et al.* 1996; Schneitz *et al.* 1997). The progeny from complementation tests were used for double-mutant analysis (described in § 3d).

Based on analysis of 60 chromosomes in the mapping population, *PFS2* showed linkage with genetic markers near the top of chromosome 2. *PFS2* mapped 1.9 and 30 centiMorgans north of RGA and GPA1, respectively. No other ovule mutant maps to this location. These data indicated that *pfs2* defines a new locus regulating ovule development.

(d) *Double-mutant analysis with* pretty few seeds2

Double mutants were evaluated to place the *PFS2* locus into existing developmental pathways (Gasser *et al.* 1998;

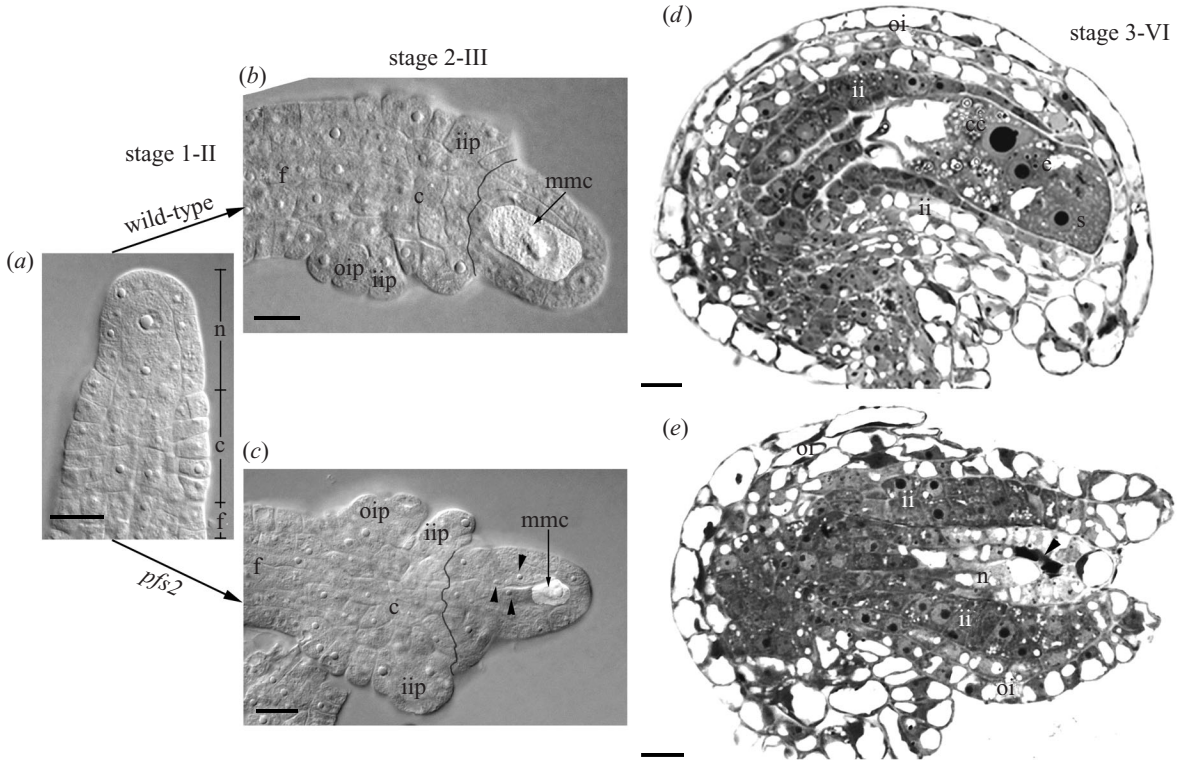

Figure 1. The anatomies of *pfs2-1* and wild-type ovules are compared. (*a*) Ovule primordia differentiate into three regions: funiculus, chalaza and nucellus. (*b*) In wild-type ovules, the inner and outer integument primordia emerged from the chalaza region. The boundary between the chalaza and nucellus is outlined. (*c*) In the *pfs2-1* mutant, the size of the megaspore mother cell was highly reduced when compared with the megaspore mother cell of wild-type ovules. Additional cells occupied this region in *pfs2-1* ovules (arrowheads). Although a boundary between the nucellus and chalaza is outlined, this boundary is not distinct as it is in wild-type ovules. (*d*) This section through the gametophyte of a wild-type ovule shows the central cell, egg cell and synergid. (*e*) In the *pfs2-1* mutant, the nucellus is still present as the gametophyte failed to develop. An aborted megaspore was found (arrowhead). In addition, the outer integument was recessed. Stages according to Schneitz *et al.* (1995). f, funiculus; c, chalaza; n, nucellus; iip, inner integument primordia; oip, outer integument primordia; mmc, megaspore mother cell; e, egg cell; s, synergid; cc, central cell; ii, inner integument; oi, outer integument. Scale bars, 5 μm.

Grossniklaus & Schneitz 1998). The stronger *pfs2-1* allele was used for double-mutant analysis.

(i) *Synergistic interaction of* pfs2 *and* ino

The *INO* gene specifies abaxial cell identity in ovules and induces asymmetric growth of the outer integument (Villanueva *et al.* 1999). In *ino* mutants, the outer integument primordium initiated, but it failed to undergo the asymmetric cell expansion found in wild-type ovules (figure 2*a*). In the *pfs2-ino* double mutants, the inner integument was lobed, bifurcating into two or more protrusions (figure 2*c*,*d*). While the nucellus remained distinct from these lobes, the megaspore mother cell was absent (figure 2*d*).

(ii) *Redundant function of* pfs2 *and* nzz *on integument development*

NZZ is required for megaspore formation (Schiefthaler *et al.* 1999; Yang *et al.* 1999), but it has been hypothesized that this locus is also involved in integument formation (Balasubramanian & Schneitz 2000, 2002). This assertion is surprising because *nzz* mutant integuments are indistinguishable from the wild-type integuments (Schiefthaler *et al.* 1999; Yang *et al.* 1999). However, *nzz* mutants exacerbated integument phenotypes in various ovule mutant backgrounds (Balasubramanian & Schneitz 2000, 2002). Integument development in *pfs2-nzz* double mutants displayed a strong ovule phenotype: integument primordia aborted shortly after formation (figure 3*b*). This demonstrates that either *NZZ* or *PFS2* activity is needed for integument development.

(iii) pfs2 *increases the penetrance of* sub

The phenotype of *sub* ovules exhibited a high degree of variability: some *sub* ovules appeared normal, whereas others were lobed (figure 4*a*). The integuments in *sub* ovules were replaced with finger-like projections (Schneitz *et al.* 1997). In the *pfs2-sub* double mutant, all of the ovules had filamentous protrusions in place of the integuments (figure 4*b*). In addition, these projections were recessed back to the chalaza in the double mutant. Putting *sub* into a *pfs2* genetic background increased the penetrance of the *sub* phenotype.

4. DISCUSSION

(a) *The* PRETTY FEW SEEDS2 *gene regulates ovule patterning*

There were three distinct phenotypes observed in *pfs2* mutants: frequent failure of gametophyte differentiation, aberrant morphogenesis of integuments and defects in the zonation of ovule primordia (figure 1, and data not shown). While the *PFS2* gene might directly regulate both ovule patterning and gametophyte formation, the data

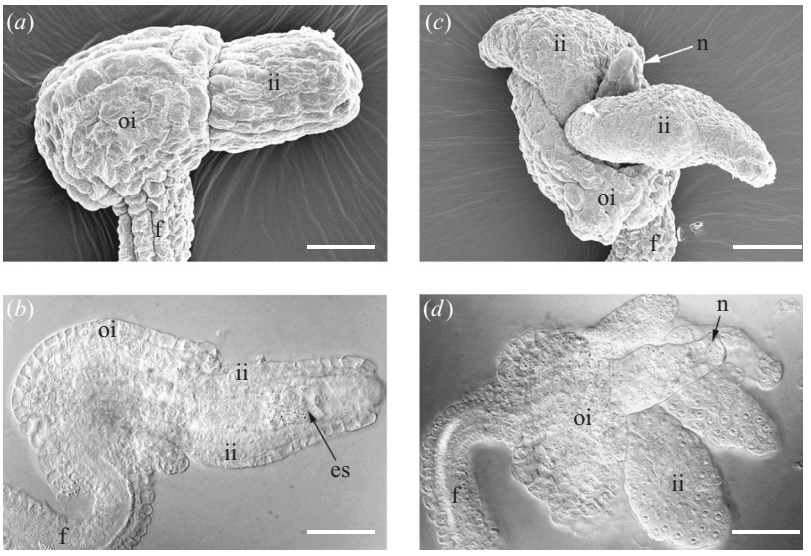

Figure 2. (*a*) In the *ino* mutant, the outer integument primordia initiated, but failed to develop fully. (*b*) The *ino* mutant ovule has a viable embryo sac. (*c*) The *pfs2-ino* double mutant had reduced growth of the outer integument, thus resembling the development of the *ino* single mutant. In addition, the inner integument bifurcated into lobes. (*d*) The embryo sac was absent in the *pfs2-ino* double mutant. The nucellus was outlined to identify this region. In addition, the inner integument split into four finger-like projections, which appear similar to the evolutionary progenitors of the inner integument. oi, outer integument; es, embryo sac; ii, inner integument; n, nucellus; f, funiculus. Scale bars, 10 μm.

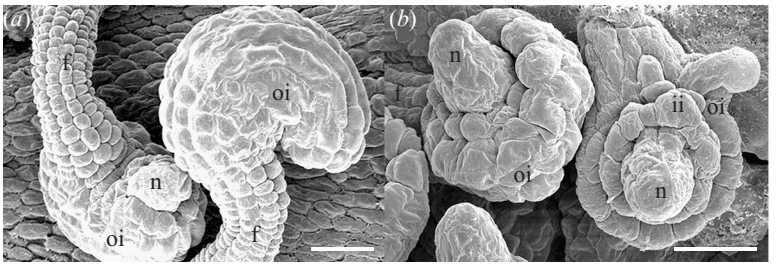

Figure 3. (*a*) In the *nzz* mutants, the external morphology was similar to that of the wild-type ovules, although they were reduced in size. (*b*) In the *pfs2-nzz* double mutant, the integument primordia initiated, but failed to develop further. The reduction in size of the inner and outer integuments led to the nucellus being exposed. The phenotype of the *pfs2-nzz* double mutants indicates that these loci function redundantly with respect to integument development. ii, inner integument; oi, outer integument; f, funiculus; n, nucellus. Scale bars, 10 μm.

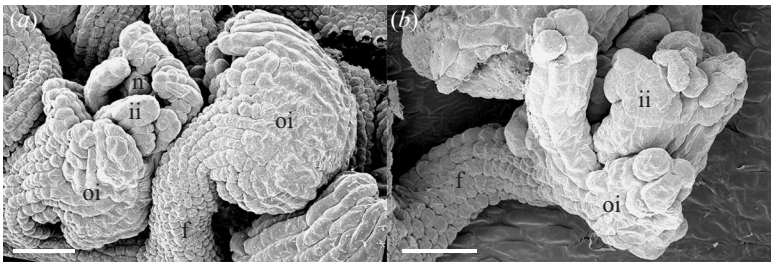

Figure 4. SEMs of *sub* and *pfs2-sub* ovules. (*a*) Integument morphogenesis was disrupted in *sub* mutants. Some of these integuments are lobed at the tips, while others are indistinguishable from those of the wild-type ovules. (*b*) Both integuments in the *pfs2-sub* double mutant had finger-like structures, which appear similar to telomes. ii, inner integument; oi, outer integument; f, funiculus; n, nucellus. Scale bars, 10 μm.

were inconsistent with this hypothesis. In *pfs2* heterozygotes (*pfs2*/+), the haploid gametophytes all had a wild-type phenotype. Because the genotypes of 50% of these gametophytes were mutant and the other 50% were wild-type, the effects on gametophyte development must result from changes in maternal development. The first observable defect in *pfs2* mutants was a shift in the boundary between the chalaza and nucellus, which moved towards the distal end of the ovule (figure 1). In fact, all *pfs2* phenotypes could derive from defects in patterning of the ovule primordia.

Double-mutant analysis indicates that the *PFS2* locus regulates the development of integument primordia (figures 2–4). Based on the phenotype of the *pfs2-nzz*

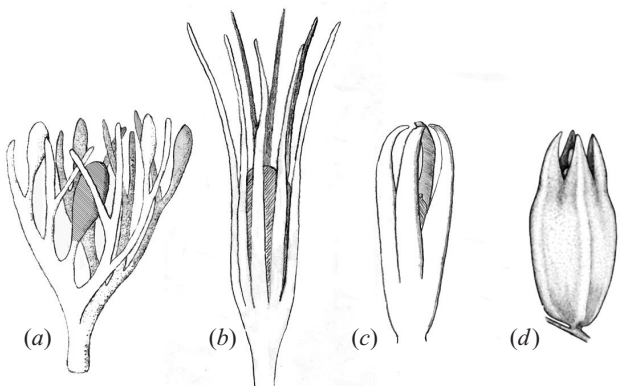

Figure 5. Various steps of integument evolution are illustrated using reconstructions from plant fossils. (*a*) *Hedeia corymbosa*. Sterile branches or telomes surround a single megasporangium, which is shaded. (*b*,*c*) As evolution proceeded, telomes fused together. This process began at the proximal end of the ovule and moved distally; (*b*) *Genomosperma kidstonii* and (*c*) *Elkinsia polymorpha*. (*d*) *Eurystoma angulare*; an ovule where telome fusion is nearly complete. Panels were drawn based on the research of Andrews (1963) and Herr (1995).

double mutant, these loci have redundant functions with respect to integument morphogenesis. In this double mutant, the inner and outer integument primordia formed, but their growth was severely retarded (figure 3). The *NZZ* locus encodes a novel protein that is reported to be essential for nucellus zonation and sporocyte formation (Schiefthaler *et al.* 1999; Yang *et al.* 1999). Balasubramanian & Schneitz (2000) proposed that this locus also regulates integument development. The early derailment of integument formation in the *pfs2-nzz* double mutant supports the hypothesis that *NZZ* and *PFS2* genes modulate integument development.

The phenotype of *sub* mutants indicates that this locus regulates integument morphogenesis (Schneitz *et al.* 1997). The *SUB* locus encodes a leucine-rich repeat kinase (Chevalier & Schneitz 2000), suggesting that this protein regulates cell-to-cell communication during integument formation, as do other genes of this class (Kobe & Deisenhofer 1994; Becraft 1998). The penetrance of the *sub* phenotype increases in the *pfs2* genetic background (figure 4). One interpretation of this result is that *PFS2* regulates one of multiple inputs to which *SUB* responds. In the absence of *SUB* and *PFS2* action, integuments became highly bifurcated and appeared similar to telomes.

The data are consistent with the *PFS2* locus forming a developmental boundary between the chalaza and nucellus by modulating one or more morphogens. Alternatively, *PFS2* might regulate the development of integument primordia or the differentiation of cells in the nucellus. Based on the results from double-mutant analysis, it is more likely that the *PFS2* locus plays a role in integument development. In *pfs2* mutants, the failure of one of these cell types to differentiate and establish a developmental domain might induce downstream effects on development. Further work will be done to determine whether the *PFS2* gene: (i) establishes a boundary between the nucellus and chalaza during ovule patterning; or (ii) directly modulates integument development in the chalaza.

(b) *Ovule evolution*

The fossil record indicates that the following events occurred during integument evolution: (i) sterilization of peripheral sporangia; (ii) reduction of branch and/or telome length and number; and (iii) fusion of telomes around a single megasporangium (figure 5). The fossils of the plants with 'integumented' ovules shown in figure 5 were found in rocks of approximately the same age (Niklas 1997). This result indicates that the evolution of the integument was a saltational event, which then led to an adaptive radiation of plants with a variety of integument morphologies; natural selection culled many variants, but the survivors were the founders of seed plants (Niklas 1997).

In order for the peripheral telomes, but not the central megasporangium, to undergo sterilization and fusion, separate developmental fields would need to be established for these structures. Signals between the megasporangium and the surrounding telomes would need to be exchanged so that both structures maintained the proper identity. Based on the evolution of the ovule, we hypothesize that one or more signals induced sterilization and fusion of the telomes.

The extensive bifurcation of integuments in the *pfs2-ino* and *pfs2-sub* double mutants revealed that these loci might generate one of the signals required for the development of integuments into a smooth tubular structure. The *INO* gene is a member of a family of genes that specify abaxial cell identity in plants (Villanueva *et al.* 1999). *INO* is active on the abaxial region of the outer integument primordia, causing the outer integument to grow asymmetrically. The *SUPERMAN*, *ABERRANT TESTA SHAPE* and *NZZ* loci have been hypothesized to act through *INO* to establish abaxial–adaxial polarity in ovules (Balasubramanian & Schneitz 2002; Meister *et al.* 2002). The phenotype of the *pfs2* mutants indicated that this locus affects the positioning of the chalaza and nucellus. Thus, the *PFS2* gene modulated proximal–distal positioning in ovules. In the absence of signals establishing proximal–distal and abaxial–adaxial polarity, integuments appear to revert to the ancestral condition: a series of lobed structures or telomes surrounding the nucellus.

Telome-like structures have also been observed in *bell1* mutants (Herr 1995). Since *bell1* ovules exhibited homeotic reversion to pistils, it has been difficult to interpret these structures. The following question remains: are the finger-like projections found in *pfs2-ino* and *pfs2-sub* double mutants equivalent to pre-integument telomes or do they simply appear similar to them? Uncoordinated growth of integument tissue might yield lobed integuments that appear similar to, but are not homologous with, telomes. In fact, there are a few species in which one of the integuments terminates in a number of wavy projections (Herr 1995). Telomes had a central vascular bundle, but the finger-like projections found in double mutants lacked this structure. While there are notable exceptions, the vasculature in most angiosperm ovules terminates in the funiculus or chalaza (Kapil & Vasil 1963). Consequently, it was not surprising that the vascular bundle was absent in the integuments of double mutants. Evaluating the function of the *PFS2* genes in basal seed plants may provide further insights into ovule evolution and address the question above.

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