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The *Arabidopsis* mutant *feronia* disrupts the female gametophytic control of pollen tube reception

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SUMMARY

Reproduction in angiosperms depends on communication processes of the male gametophyte (pollen) with the female floral organs (pistil, transmitting tissue) and the female gametophyte (embryo sac). Pollen-pistil interactions control pollen hydration, germination and growth through the stylar tissue. The female gametophyte is involved in guiding the growing pollen tube towards the micropyle and embryo sac. One of the two synergids flanking the egg cell starts to degenerate and becomes receptive for pollen tube entry. Pollen tube growth arrests and the tip of the pollen tube ruptures to release the sperm cells. Failures in the mutual interaction between the synergid and the pollen tube necessarily impair fertility. But the control of pollen tube reception is not understood. We isolated a semisterile, female gametophytic mutant from *Arabidopsis thaliana*, named *feronia* after the Etruscan goddess of fertility, which impairs this process. In the *feronia* mutant, embryo sac development and pollen tube guidance were unaffected in all ovules, although one half of the ovules bore mutant female gametophytes. However, when the pollen tube entered the receptive synergid of a *feronia* mutant female gametophyte, it continued to grow, failed to rupture and release the sperm cells, and invaded the embryo sac. Thus, the *feronia* mutation disrupts the interaction between the male and female gametophyte required to elicit these processes. Frequently, mutant embryo sacs received supernumerary pollen tubes. We analysed *feronia* with synergid-specific GUS marker lines, which demonstrated that the specification and differentiation of the synergids was normal. However, GUS expression in mutant gametophytes persisted after pollen tube entry, in contrast to wild-type embryo sacs where it rapidly decreased. Apparently, the failure in pollen tube reception results in the continued expression of synergid-specific genes, probably leading to an extended expression of a potential pollen tube attractant.

Key words: *Arabidopsis thaliana*, Double fertilization, Female gametophyte, *feronia*, Pollen tube invasion, Pollen tube reception, Sperm cell release, Supernumerary pollen tubes, Synergid degeneration

INTRODUCTION

The life cycle of plants alternates between two generations, represented by the diploid sporophyte and the haploid gametophyte (Raven et al., 1999). The sporophyte gives rise to sexually differentiated spores through meiosis. These spores divide mitotically and develop into gametophytes, whose main function is to produce the gametes. The fusion of male and female gametes establishes the next sporophytic generation. In angiosperms the male gametophyte is represented by the pollen, which consists of only three cells: a vegetative cell harbouring the two sperm cells. The female gametophyte (FG), also referred to as the embryo sac, typically comprises seven cells: the egg cell, which is flanked by two synergids, the central cell, and the three antipodals (Fig. 1A) (Grossniklaus and Schneitz, 1998). The FG is enclosed within the ovule inside the carpel and, therefore, inaccessible for the non-motile male gametophytes. Thus, angiosperms acquired a specialised form of sperm cell delivery: the pollen grain hydrates on the papillar cells of the stigma, germinates, and forms a pollen tube (PT) in which the two male gametes are transported to their destination. The PT grows through the stylar transmission tract, is attracted to the ovule and guided into the micropyle, where the sperm cells are delivered to the FG (Fig. 1B). During double fertilization one sperm cell fuses with the egg cell, which gives rise to the zygote, while the second sperm cell fertilizes the central cell to induce endosperm formation.

Several interactions between the pollen and the female reproductive tissues are required to control PT growth through the pistil and during double fertilization. They include interactions of the pollen with both sporophytic pistil tissue and the gametophytic cells of the FG. Over the last few years components mediating pollen-pistil interactions have been identified (reviewed by Johnson and Preuss, 2002; Pruitt, 1999; Wilhelmi and Preuss, 1999). In contrast, the knowledge about signals exchanged between the male and the female

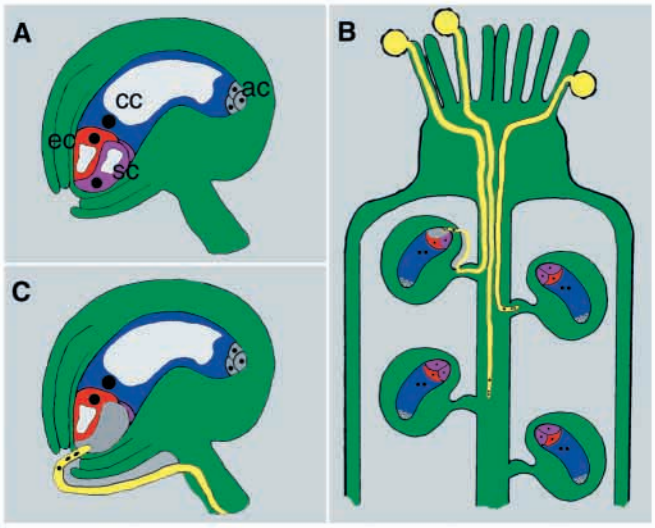


Fig. 1. (A) Schematic drawing of a mature female gametophyte (FG) enclosed within the ovule. The FG consists of the two synergid cells (sc), an egg cell (ec), the central cell (cc) and three antipodal cells (ac). (B) Path of the pollen tube (PT) through the style and transmitting tissue towards the ovules. (C) PT entering the FG through the receptive synergid.

gametophyte is very limited. Genetic experiments clearly demonstrated that the FG controls PT guidance to the embryo sac (Higashiyama et al., 1998; Hülskamp et al., 1995b; Ray et al., 1997), but the nature of the guiding cue is unknown. Recent investigations in *Torenia fournieri* revealed that the synergids control PT guidance, because the ablation of both synergids completely abolished PT attraction to the ovule (Higashiyama et al., 2001). The synergids do not only control PT guidance, they also play an essential role in the process of double fertilization (Russell, 1992). The PT is guided into the micropyle where it enters the embryo sac by growing into one of the synergids (Fig. 1C), where PT reception occurs. PT reception involves the arrest of PT growth, the rupture of the PT tip, and the release of the sperm cells, which are targeted to the egg and the central cell, respectively.

In many species the receptive synergid degenerates before the PT reaches the FG (reviewed by Kapil and Bhatnagar, 1975; Russell, 1992). Only one synergid is susceptible for signals induced by pollination or derived from the approaching PT. It is not known how the receptive synergid is selected. Synergid degeneration is considered to be essential for fertilization (Drews and Yadegari, 2002; Jensen and Fisher, 1968; van Went and Willemse, 1984). Because PT reception and double fertilization are rapid processes that involve inaccessible cells, studies about male-female gametophytic interactions have been restricted to ultrastructural investigations of pollen tube arrival, synergid degeneration and sperm cell release (reviewed by Kapil and Bhatnagar, 1975; Raghavan, 1997; Russell, 1992). The identification of mutants with defects in PT reception and double fertilization is necessary to gain insights into the genetic control and the molecular basis of these processes. In the last years screening strategies have been developed in *Arabidopsis thaliana* to identify mutants with female gametophytic defects (Christensen et al., 1998; Howden et al., 1998; Moore et al., 1997; Shimizu and Okada, 2000). Although most mutations identified to date cause defects in embryo sac development (Christensen et al., 1998; Moore et al., 1997), some appear to be implicated in PT guidance and reception (Christensen et al., 2002; Shimizu and Okada, 2000). For instance, the *Arabidopis* mutant *gfa2* fails in synergid degeneration (Christensen et al., 2002). The PT is still attracted to the FG but the embryo sac remains unfertilized. Synergid degeneration is, therefore, not required for PT guidance but for PT reception. The *GFA2* gene encodes a member of the DnaJ protein family and is thought to function as a chaperone in mitochondria. How the GFA2 protein controls cell death in the synergid is unknown.

We report the comprehensive phenotypic characterization of the *Arabidopsis* female gametophytic mutant *feronia* (*fer*/+). The mutant is named after the Etruscan goddess of fertility, because one half of the ovules remain unfertilized. PT reception is impaired in the *feronia* mutant, although embryo sac development is unaffected and synergid specification and differentiation appear to be normal. In *feronia* mutant FG, the PT continues to grow and invades the FG instead of arresting its growth and rupturing to release the sperm cells. Our analysis suggests that the *feronia* mutation disrupts the interaction between the male and the female gametophyte and, thus, defines a novel signalling process required for PT reception.

MATERIALS AND METHODS

Plant material and growth conditions

The *feronia* mutant was isolated from a collection of transposants generated by mutagenesis using enhancer detector and gene trap *Ds* elements as described previously (Moore et al., 1997; Sundaresan et al., 1995). The insertional mutants were generated using the *Ac/Ds*-transposon system of Sundaresan et al. (Sundaresan et al., 1995) in *Arabidopsis* plants of the Landsberg *erecta* (L*er*) ecotype. The pollen tube marker EC3 and three enhancer detector lines with synergid-specific GUS activity were kindly provided by R. Baskar. The plants were grown on soil ED73 (Universal Erde, Germany) in an indoor growth facility with 70% relative humidity and a day-night cycle of 16 hours light at 21°C and 8 hours darkness at 18°C. For crosses with dehiscent anthers closed flower buds were emasculated 24-48 hours before pollination. For limited pollination a restricted amount of pollen was transferred using an eyelash. Tetraploid plants carrying the mutant *fer* allele were obtained by crossing and back-crossing *feronia* to tetraploid L*er* plants and were maintained through selfing. The ploidy level was confirmed using a Ploidy Analyser (Partec, Münster, Germany) as described (Matzk et al., 2000).

Molecular biology

Non-radioactive Southern blots using a digoxigenin-labelled probe (base pairs 91 to 440 of the Ds element, accession number AF433043) were performed according to the manufacturer's instructions. TAIL-PCR (Liu and Whittier, 1995) to isolate the sequences flanking Ds was performed as described previously (Grossniklaus et al., 1998). Primers to amplify the molecular marker InDel D03, which shows a 6 bp size difference between Ler and Columbia (Col) were NHP168 (5′-GGAGAGTAATCAGCAGCTGAG-3′) and NHP169 (5′-GGAG-AGTAATCAGCAGCTGAG-3′).

Cleared whole-mount preparations and histology

For phenotypic characterization, seeds were cleared following the protocol of (Yadegari et al., 1994). GUS assays were performed as described previously (Vielle-Calzada et al., 2000). Specimens were observed using a Lecia DMR microscope (Leica Microsystems,

Bensheim, Germany) under bright-field Normarski optics. For preparation of semi-thin sections, plant siliques were fixed overnight in 3.7% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) on ice. Specimens were dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, 95%, 3× 100%) and transferred into Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. The tissue was sectioned at 8 μm thickness on a Leica RM 2145 microtome. After staining with 0.05% Toluidine Blue, sections were observed under bright-field optics using a Leica DMR microscope.

For ultrastructural analysis silques were cut into 1 mm pieces and fixed on ice for 4 hours with 2.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.0. After three washes with sodium phosphate buffer the siliques were post-fixed in 2% buffered osmium tetroxide, washed and dehydrated in an acetone series (50%, 70%, 90%, 2× 100%) and infiltrated with Spurr's epoxy resin. Siliques were opened and ovules embedded in Spurr's epoxy resin between Teflon-coated microscope slides and polymerized at 60°C. Ultrathin sections were contrasted with 1% uranyl aceteate and Sato's lead solution. Specimens were examined in a Hitachi H 7000 TEM (Hitachi Ltd., Tokyo, Japan) at 60 kV.

Fluorescence staining of pollen tubes

For pollen tube staining, opened siliques were fixed overnight in Lavdowsky's FAA (1.5% formaldehyde, 2% acetic acid and 30% ethanol) at 4°C and washed in an alcohol series (70%, 50% 30% 10%), for 10 minutes each. Tissue was softened with 10% chloralhydrate at 60°C for 10 minutes, rinsed twice with sodium phosphate buffer (100 mM, pH 7) and then in 5 M NaOH at 60°C, for 5 minutes. Pollen tubes were stained with 0.1% Methyl Blue (certified for use as Aniline Blue; Sigma, St. Louis, USA). Stained samples were observed using a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany) equipped with an epifluorescence UV-filter set (excitation filter at 365 nm, dichroic mirror at 395 nm, barrier filter LP at 420 nm). Confocal observations of stained samples were made using a Leica TCS-SP microscope. The excitation wavelength was 430 nm and the spectral detection window was set as 450-580 and 700-800 nm. Images were acquired and processed using the Leica Confocal Software, Version 2.0.

Image processing

All images were processed for publication using Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, USA).

RESULTS

The *feronia* mutation causes female semisterility

With the goal of isolating mutants of *Arabidopsis thaliana* that display defects in female gametophyte development or function, we performed insertional mutagenesis screens (J. M. and U. G., unpublished) (Moore et al., 1997; Page and Grossniklaus, 2002). We used the *Ac/Ds* transposon system developed by Sundaresan et al. (Sundaresan et al., 1995), where the *Ds* element carries an *NPTII* gene conferring kanamycin resistance. Plants carrying a transposed *Ds* element were subjected to a two-step screen (Moore et al., 1997; Page and Grossniklaus, 2002): progeny of plants displaying semisterility (seed set <70%) were tested for segregation ratio distortion of the kanamycin resistance gene. Segregation ratio distortion is indicative of a gametophytic defect as opposed to other potential causes of semisterility, e.g. reciprocal translocations or poorly penetrant sporophytic steriles (Drews and Yadegari, 2002; Moore et al., 1997).

The gene-trap line *feronia* had reduced seed set that varied

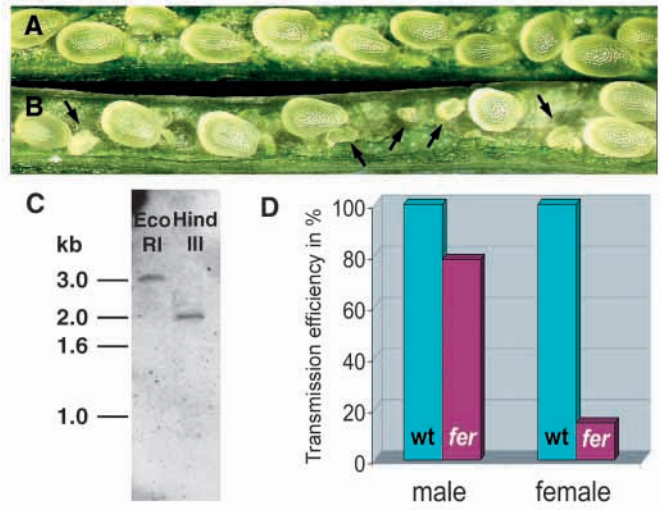


Fig. 2. The *feronia* mutant is semisterile and shows reduced transmission through the female gametophyte. (A) Wild-type siliques show full seed set. (B) In *feronia* siliques only about half of the ovules develop into seeds, the others remain unfertilized (arrows). (C) Southern blot using *Ds* 5′ end-specific probe indicates a single *Ds* insertion in the *feronia* mutant. The observed fragment sizes are consistent with the expected sizes after restriction with *Eco*RI (3.2 kb) and *Hin*dIII (2.0 kb). (D) Transmission efficiency (TE) of the kanamycin resistance gene, which is tightly linked to *feronia*, through wild-type and *feronia* gametophytes [TE=(kanamycin resistant seedlings)/(kanamycin sensitive seedlings); progeny of reciprocal out-crosses to the wild type]. In *feronia* the transmission through the male gametophyte is reduced to 78.5% and through the female gametophyte to 14.5%.

from 45% to 60% in individual siliques. Three phenotypic classes of ovules/seeds were observed: on average (n>2000) 50% of the seeds were normal, 1% aborted early during seed development, and 49% of the ovules remained unfertilized and senesced (Fig. 2A,B). In a population of 446 plants the kanamycin resistance gene co-segregated with semisterility, suggesting that the *Ds* element is tightly linked to the *feronia* mutation. The segregation of kanamycin resistance and the result of a Southern blot hybridized with a *Ds*-specific probe (Fig. 2C) strongly suggest that a single *Ds* element is present in *feronia*. Thus, the kanamycin resistance gene could be used as a marker for the mutant *feronia* allele, greatly facilitating subsequent genetic analyses. However, reversion of the *feronia* phenotype through excision of the *Ds* element was unsuccessful, as was complementation with a genomic fragment containing the disrupted gene, indicating that the *feronia* mutant is not tagged.

To determine the map position of *feronia*, we isolated genomic sequences flanking the tightly linked *Ds* element by thermal asymmetric interlaced PCR (Liu et al., 1995). Comparison of the PCR-product with the *Arabidospis* genome sequence allowed us to position the *Ds* element on BAC F26O13. To confirm this genetic map position based on the *feronia* phenotype, a molecular marker (InDel D03) on this BAC was designed using an InDel between the Columbia (Col) and Landsberg *erecta* (L*er*) sequence (Jander et al., 2002). No recombinants were detected between this marker and *feronia* among 62 F_2 individuals derived from a cross between *fer*/+

(L*er*) and Col confirming that *feronia* maps on chromosome 3 at approximately 72 centiMorgans.

To confirm that the semisterile phenotype of *feronia* was indeed caused by a gametophytic defect we analyzed the segregation ratio of the kanamycin resistance gene in its progeny. For a fully penetrant gametophytic mutation affecting specifically the female gametophyte, we expect a segregation ratio of mutant to wild-type plants of 1:1, as half of the male gametophytes carry the mutation in a heterozygote but only wild-type female gametophytes are functional. Alternatively, reciprocal chromosome translocations can lead to semisterility in both the male and female sex (Ray et al., 1997). However, while wild-type and semisterile plants are expected to segregate 1:1 in the progeny of a plant carrying a reciprocal translocation, markers present on the chromosomes involved in the translocation segregate in a Mendelian fashion. The segregation ratio of the kanamycin resistance gene in the progeny of selfed *feronia* (*fer*/+) plants was 1.04:1.00 resistant to sensitive seedlings in the F_3 (n=501), and 1.18:1.00 in the F_4 generation (n>1000). The distorted segregation ratio of the associated kanamycin resistance gene and the fact that no pollen abortion was observed in *fer*/+ plants (data not shown) strongly suggests that semisterility in *feronia* is caused by a gametophytic defect and not by a reciprocal translocation or some other gross chromosomal rearrangement.

feronia is a loss-of-function mutation predominantly affecting the female gametophyte

While the segregation ratio distortion observed in *fer*/+ plants is close to the expected distortion for a sex-specific gametophytic mutation, it does not exclude the possibility that both sexes are partially affected. To investigate the respective contribution of male and female gametophytic defects, we determined the transmission efficiency of the *feronia* mutant allele through either sex by reciprocal out-crosses to the wild type. The analysis of the transmission efficiency of the kanamycin resistance gene (Fig. 2D) demonstrated a slight reduction through the male (TE_{male}=78.5%, n=930) but a strong reduction through the female gametophyte (TE_{female}=14.5%, n=749). Despite a significant transmission of the kanamycin resistance gene through both gametophytes, homozygous plants were never recovered, indicating that the mutation causes zygotic lethality.

In order to make any conclusions about the wild-type function of a gene, it is important to determine whether a mutation is recessive or dominant to the wild type. Because dominance and recessiveness are defined as interactions between two alleles in the same nucleus, it is not possible to investigate this interaction in haploid gametophytes. Therefore, we generated tetraploid plants, which produce diploid gametophytes, by repeatedly crossing *feronia* to wild-type tetraploids. Siliques of tetraploid plants potentially carrying between one and three *feronia* alleles were analyzed microscopically for the *feronia* phenotype (see below). A plant where approximately 50% of the relevant ovules showed the *feronia* phenotype [50 wild type, 42 *feronia*, 36 arrested ovules as typical of tetraploid L*er* plants (Grossniklaus et al., 1998)] was analyzed further. As shown in Table 1, such a *feronia* segregation ratio is expected in either a simplex tetraploid with *feronia* being dominant (fer^D/+/+/+) or a triplex tetraploid with *feronia* being recessive (*fer*/*fer*/*fer*/+). While the *feronia* phenotype is expected to segregate in a similar ratio among the ovules in these two cases, the associated *Ds* element is expected to segregate very differently among their progeny. As shown in Table 1, the segregation pattern of the *Ds* element is consistent with a triplex tetraploid parent, strongly suggesting that *feronia* is a recessive, loss-of-function mutation. Therefore, it should be possible to identify insertional alleles in the area to facilitate the molecular isolation of the gene. Taken together these segregation and transmission analyses strongly suggest that the *feronia* mutation is recessive, affects predominantly the female gametophyte, and causes zygotic lethality when homozygous, consistent with the small percentage of early aborting seeds that we observed.

In *feronia* mutant embryo sacs pollen tube growth does not arrest and the sperm cells are not released

The arrested ovules in the *feronia* (*fer*/+) mutant indicated a defect in megagametogenesis, PT guidance or double fertilization. To determine which process is impaired in the *feronia* mutant we analyzed the embryo sacs in cleared whole mounts of mutant and wild-type siliques at different stages. In all *feronia* (*fer*/+) pistils analyzed, the FG developed normally to an eight nucleate embryo sac of the *Polygonum*-type that is typical for *Arabidopsis* (Fig. 3A) (Christensen et al., 1997; Schneitz et al., 1995). When analyzing pistils from wild-type and *feronia* (*fer*/+) plants 24 hours after pollination (HAP), the embryo sacs of all wild-type ovules and of one half of the *feronia* (*fer*/+) ovules had initiated the formation of free nuclear endosperm (Fig. 3B). The remaining *feronia* embryo sacs were unfertilized. The egg cell and central cell nucleus but no synergid nuclei were detectable in unfertilized FGs

Table 1. Determination of recessiveness or dominance of the *feronia* allele in tetraploid *feronia* plants

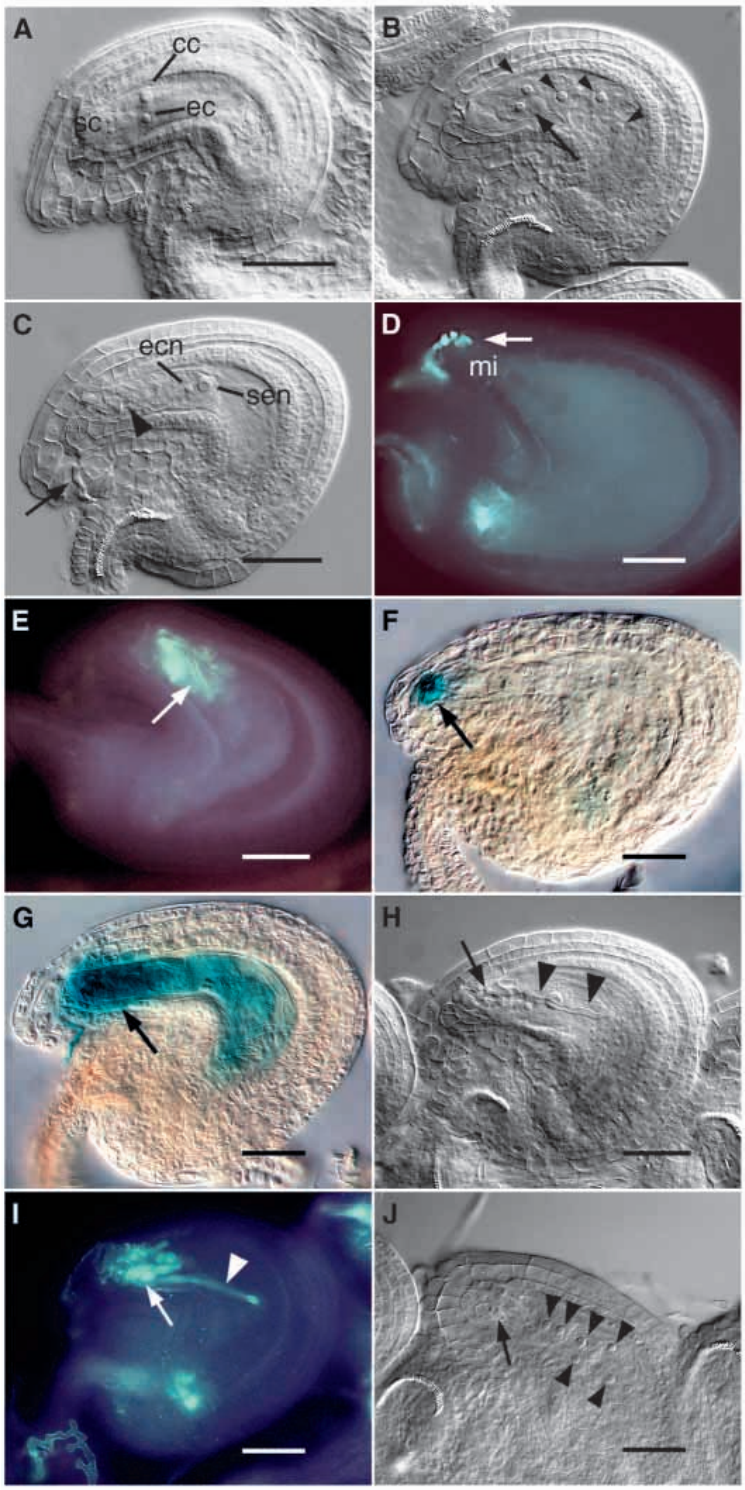
	Phenotype (*fer*:wt)			Segregation Kan^{res}: Kan^{sen}		
Genotypes	Expected	Observed	*P*	Expected	Observed	*P*
Simplex, rec (*fer*/+/+/+)	2:90	42:50	0	125:50	174:1	0
Simplex, dom (fer^D/+/+/+)	44:48	42:50	0.68	85:90	174:1	0
Duplex, rec (*fer*/*fer*/+/+)	18:74	42:50	0	166:9	174:1	0.01
Duplex, dom (fer^D/fer^D/+/+)	74:18	42:50	0	148:27	174:1	0
Triplex, rec (*fer*/*fer*/*fer*/+)	48:44	42:50	0.21	174:1	174:1	1
Triplex, dom (fer^D/fer^D/fer^D/+)	90:2	42:50	0	174:1	174:1	1

The observed ratio of the invading PT phenotype (*fer*:wt) and the segregation ratio (Kan^{res}:Kan^{sen}) only fit the expected values for a triplex recessive genotype. Expected values have been calculated according to Burnham (Burnham, 1962) using the transmission data of diploid *feronia* plants and an estimated frequency of double reduction α=0.1. Null hypothesis is consistent with the observed data for P>0.05. Data represent the results for one individual plant. Similar data have also been obtained for a second tetraploid plant (data not shown).

indicating a defect in the synergids or pollen tube reception (Fig. 3C). Pollen tube guidance was not affected in the mutant FGs, because PTs approached normally and entered the FG through the micropyle (Fig. 3C).

We consistently observed a tangled structure in the micropylar area of unfertilized mutant FGs, presumably formed by the PT invading the FG (Fig. 3C). To examine pollen reception in *feronia* (*fer*/+) we dissected pistils of both mutant and wild-type plants and stained them with Aniline Blue to visualize the PT within the ovule. In all wild-type and the fertilized *feronia* (*fer*/+) ovules the growth of the PT ceased at the micropylar end of the embryo sac (Fig. 3D). In the unfertilized *feronia* (*fer*/+) ovules there was a strong fluorescence signal from a coiling PT in the micropylar area of the embryo sac (Fig. 3E).

Taken together these results suggest that megagametogenesis and pollen tube guidance are not affected in the *feronia* mutant but double fertilization is. PT growth was not arrested in *feronia* mutant FGs and the PT continued to grow, coil around and entangle itself within the micropylar area (referred to hereafter as the 'invading PT phenotype'). Since the PT is a paradigm for polarized tip growth (Hepler et al., 2001), it is likely that the tip of the invading PT stays intact within *feronia* mutant embryo sacs and that fertilization does not take place because the PT fails to rupture and to release the sperm cells.



A defect in the embryo sac causes the aberrant behaviour of the pollen tube

The analyses of the transmission efficiencies through both gametophytes of the *feronia* (*fer*/+) mutant indicated a strong defect in the FG, and a weaker defect in the male gametophyte (Fig. 2B). Since the genetic data supported a female gametophytic defect but the phenotype is manifest in the male gametophyte, we asked to what extent the two gametophytes contribute to the invading PT phenotype. To this aim, we performed reciprocal crosses between wild-type and *feronia* (*fer*/+) plants and analyzed the pistils 48 and 68 HAP.

When we pollinated wild-type plants with wild-type pollen, we did not observe the invading PT phenotype. But when wild-type pistils were pollinated with *feronia* (*fer*/+) pollen we noticed that PTs occasionally invaded the FG (2 out of 110 at 48 HAP, 2 out of 229 at 68 HAP, see Table 2). However, in these few cases the PT coiling

Fig. 3. Invading PT phenotypes in the *feronia* mutant. (A-C,H,J) Cleared whole-mount preparations. (D,E,I) Ovules stained with Aniline Blue to visualize the PT. (F,G) Crosses to PT marker line expressing the β-glucuronidase (GUS) gene. (A) Mature FGs of wild-type and *feronia* (*fer*/+) plants before fertilization are indistinguishable from each other and contain synergid cells (sc), egg cell (ec) and central cell (cc). (B) Fertilized wild-type ovules at 24 HAP that contain an elongated zygote (arrow) and free nuclear endosperm (arrowheads). (C) Unfertilized *feronia* (*fer*/+) ovule at 24 HAP. The secondary endosperm nucleus (sen) of the central cell and the egg cell nucleus (ecn) are visible. The pollen tube (arrow) enters the ovule but forms an entangled structure (arrowhead) within the FG. (D) Wild-type ovule after fertilization. The PT (arrow) terminated in the micropylar area (mi) of the embryo sac. (E) *feronia* mutant in which the PT invades the micropylar area (arrow). (F) In wild-type ovules the GUS-activity of the PT remains restricted to the micropylar area of the FG (arrow). (G) In mutant FG the GUS signal is observed in the entire micropylar part of the FG. Owing to the strong GUS activity, the stain diffused into the central cell. (H,I) The PT enters the central cell (arrowheads) after coiling in the micropylar area (arrow). (J) Formation of free nuclear endosperm (arrowheads) after the PT has invaded the FG (arrow). Scale bars: 30 μm.

Table 2. Observed frequencies of invading pollen tube phenotypes

	Number of ovules	Normal reception		Invading PT phenotype					Unfertilized
		Total	sn PTs	Total	sn PTs	PT in CC	end form	emb form	Total
48 HAP									
L*er* × L*er*	122	113	n.d	0	–	–	–	–	9
L*er* × *fer*/+	130	110	2	2	0	0	0	0	18
fer/+ × L*er*	199	93	1	91	9	1	7	1	15
fer/+ × *fer*/+	276	121	2	114	13	3	13	0	41
68 HAP									
L*er* × L*er*	208	187	1	0	–	–	–	–	21
L*er* × *fer*/+	254	229	2	2	0	0	0	0	23
fer/+ × L*er*	301	152	1	133	12	3	11	6	16
fer/+ × *fer*/+	363	167	2	169	18	8	17	7	27

Observed phenotypes in crosses between Landsberg *erecta* (L*er*) wild-type plants and *feronia* (*fer*/+) 48 and 68 hours after pollination (HAP).
sn PTs: arrival of supernumerary pollen tubes; PT in CC: pollen tube growth into the central cell; end form: formation of endosperm; emb form: formation of embryo-like structures.

was not as pronounced as described above (data not shown). In contrast, pollinated *feronia* (*fer*/+) pistils always contained about 50% ovules invaded by the PT, regardless of whether the pollen was derived from wild-type or *feronia* plants (see Table 2). The frequency of PT invasion corresponded to the observed semisterility. In a similar experiment, we crossed *feronia* and wild-type pistils with pollen of a marker line expressing the GUS reporter gene (Jefferson et al., 1987) in the PT (R. Baskar and U. G., unpublished). In the FG of all wild-type and half of the *feronia* (*fer*/+) ovules the PT terminated in the synergid (Fig. 3F). In the unfertilized *feronia* FGs pollen tube growth failed to arrest resulting in an intense GUS staining of the coiled PT in the micopylar area of the mutant (Fig. 3G).

The results of these crosses strongly suggest that the invading PT phenotype in the *feronia* (*fer*/+) mutant is exclusively determined by the genotype of the FG. Thus, the *feronia* mutation affects a communication process between male and female gametophytes required for PT reception and, consequently, double fertilization.

Invading pollen tubes cause secondary embryo sac phenotypes

During the phenotypic characterization of the *feronia* mutant we frequently observed invading PTs growing into the central cell (Fig. 3H,I). In other cases, we noticed the formation of endosperm or embryo-like structures, although the PT was coiling at the micropylar end of the FG (Fig. 3J). We determined the frequency of these phenotypes in cleared whole-mount preparations. To determine whether the ovules with formation of endosperm and embryo-like structures were the homozygous *feronia* progeny that we expect to be zygotic lethal, we analyzed pistils of reciprocal crosses between *feronia* (*fer*/+) and wild-type plants 48 and 68 HAP.

The frequency of PTs entering the central cell in mutant *feronia* FG increased from 1-2% at 48 HAP to 3-5% at 68 HAP (see Table 2). The phenotype occurred regardless of whether mutant pistils were pollinated with pollen from wild-type or *feronia* plants. However, the number of PTs proceeding into the central cell was slightly higher when mutant pollen was used (see Table 2). In contrast to the tangled growth at the micropylar end, PTs that entered the central cell grew straight to the chalazal end of the FG (Fig. 3H,I). In some cases the PT proceeded to the chalazal part of the embryo sac and turned back to grow towards the micropylar tip (data not shown).

In about 10% of the invaded FGs the secondary endosperm nucleus of the central cell started to divide. The frequency was independent of the pollen source, wild-type or *feronia*, and did not change from 48 HAP to 68 HAP. In some cases we observed the formation of embryo-like structures (Table 2), which always arrested after a few cell divisions (data not shown). Embryo initiation was observed more frequently at 68 HAP, which might be the result of the delay of embryo development compared to endosperm formation (Faure et al., 2002; Mansfield and Briarty, 1991).

In summary, the occurrence of PTs proceeding into the central cell and the formation of endosperm or embryo-like structures after PT invasion is independent of the pollen genotype and also occurs when wild-type pollen is used. Therefore, these seeds are not homozygous for the *feronia* mutation, and these phenotypes solely depend on the genotype of the female gametophyte. It is likely then that coiling pollen tubes occasionally burst and release sperm cells that effect a single fertilization event. The ability of the egg cell and the central cell to initiate cell and nuclear divisions, respectively, demonstrates that the female gametes in *feronia* are functional after PT invasion.

Mutant *feronia* embryo sacs attract supernumerary pollen tubes

It is the common view that ovules in *Arabidopsis* receive only one PT (Hülskamp et al., 1995a; Hülskamp et al., 1995b; Shimizu and Okada, 2000). Nevertheless, in hand-pollinated wild-type pistils we observed five out of 526 ovules in which supernumerary PTs approached and entered the FG (see Table 2). These ovules were delayed in both embryo and endosperm development (data not shown) suggesting that abnormal FGs permitted additional PTs to approach. However, in the *feronia* mutant we consistently observed about 10% of the invaded ovules attracting two or more PTs (Fig. 4A,B, Table 2). To determine whether the PTs approached the micropyle simultaneously or sequentially we investigated PT reception in hand pollinated *feronia* (*fer*/+) plants at 12 and 24 HAP. At 12 HAP it was difficult to discriminate mutant and wild-type FGs as only a few ovules already exhibited the invading PT

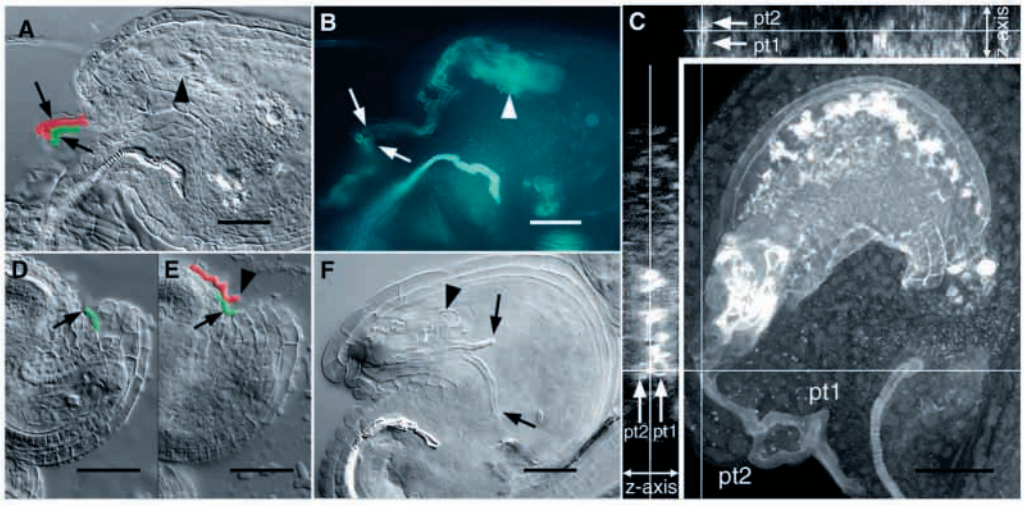


Fig. 4. Supernumerary PTs enter mutant *feronia* FGs. (A,D-F) Cleared whole-mount preparations. (B) Ovule stained with Aniline Blue to visualize the PT. (C) Confocal micrograph with transections in z-axis. A and B show the same ovule in the same focal planes. At least two PTs (arrows, PT1 false coloured in red, PT2 in green) enter the ovule at the micropyle (arrowhead). (C) Two pollen tubes (pt1, pt2) enter the ovule and grow through the micropyle towards the embryo sac. The top and the side panel show the transections of the confocal image stack in z-axis along the lines shown in the main panel. Arrows in the transections mark the two PTs reaching the FG. (D,E) Successive arrival of PTs at ovules at 12 HAP. (D) The first PT (arrow, false coloured in green) has already entered the micropyle. (E) Different focal plane of the same ovule. The second PT approaches the micropyle (arrowhead, false coloured in red). The arrow indicates the first PT entering the ovule. (F) Two PTs (arrows) have invaded (arrowhead) and entered the FG and proceeded into the central cell (cc). This specimen was derived from a tetraploid *feronia* plant. Scale bars: 30 μm.

phenotype. Nevertheless, in three of 248 ovules analyzed an additional PT had entered the micropyle, but in eleven ovules a second or third PT grew towards the micropyle (Fig. 4D,E). These numbers correlate with the observed frequency of ovules receiving supernumerary PTs at later time points. In about 10% (12 out of 113) of invaded ovules additional PTs had entered the micropyle at 24 HAP, and similar frequencies were observed at 48 HAP and 68 HAP (Table 2).

We used confocal laser scanning microscopy to follow the path of Aniline Blue-stained PTs to test whether the additional PTs that entered the micropyle proceeded towards the FG. We identified two PTs growing within the micropyle until they reached the embryo sac (Fig. 4C). PT coiling did usually not allow identification of individual PTs within the FG. However, in two whole-mount preparations, we clearly observed two PTs invading the embryo sac and proceeding into the central cell (Fig. 4F).

These experiments suggest that ovules containing mutant *feronia* FGs continue to attract additional PTs after they received the first PT. These supernumerary PTs are able to enter the micropyle, proceed towards the FG, and can potentially invade the embryo sac.

Synergids of *feronia* embryo sacs are normally specified and differentiated

In *feronia*, PT growth failed to arrest and the PTs did not rupture to release the sperm cells after entering the embryo sac. This would suggest that a communication process between male and female gametophytes is disrupted, which could either be a direct effect of disrupting a signalling process between the synergid and the pollen tube, or a secondary effect due to the abnormal development or differentiation of the synergids. To address the question of whether PT reception failed because the synergids had developed abnormally we crossed *feronia* into three independent enhancer detector lines expressing the GUS reporter gene specifically in the synergids (R. Baskar and U. G., unpublished).

In the wild type, the GUS signal was detectable after cellularization of the FG (Fig. 5A) and persisted in both synergids until fertilization. After fertilization the GUS signal decreased and became undetectable about 24 to 36 HAP (Fig. 5B). We tested whether the reduction in GUS expression was individually induced by the entry of the PT, or whether it was induced in all ovules by a general mechanism, e.g. a certain time after pollination. Therefore, we pollinated wild-type pistils with a limited number of pollen grains, so that only a few ovules were fertilized. GUS activity was readily detected in both synergids of unfertilized ovules, but was drastically reduced in ovules where a PT had entered the micropyle. This indicates that PT entry or fertilization induces the rapid decrease of the signal. In *feronia* mutants (*fer*/+) the GUS expression pattern of the three markers was indistinguishable from wild-type until fertilization (Fig. 5C). In fertilized ovules GUS activity disappeared within 24 HAP but it persisted in mutant *feronia* FGs (Fig. 5D).

The analysis of the enhancer detector lines revealed that *feronia* synergids were normally specified and differentiated. After PT entry, GUS activity disappeared in fertilized ovules harbouring wild-type gametophytes but persisted in mutant embryo sacs after PT invasion and in unfertilized wild-type embryo sacs, suggesting that the decrease of GUS activity depends on PT rupture and/or sperm release.

Degeneration of the receptive synergid appears normal at the ultrastructural level

The PT normally enters the embryo sac through one synergid. In *Arabidopsis* it was reported that this receptive synergid shows signs of degeneration before or just around the time of PT entry (Faure et al., 2002; Murgia et al., 1993). Synergid degeneration is considered to be necessary for PT rupture and sperm cell release in some species (Drews and Yadegari, 2002; Jensen and Fisher, 1968; van Went and Willemse, 1984). We investigated whether in the *feronia* mutant the PT really entered

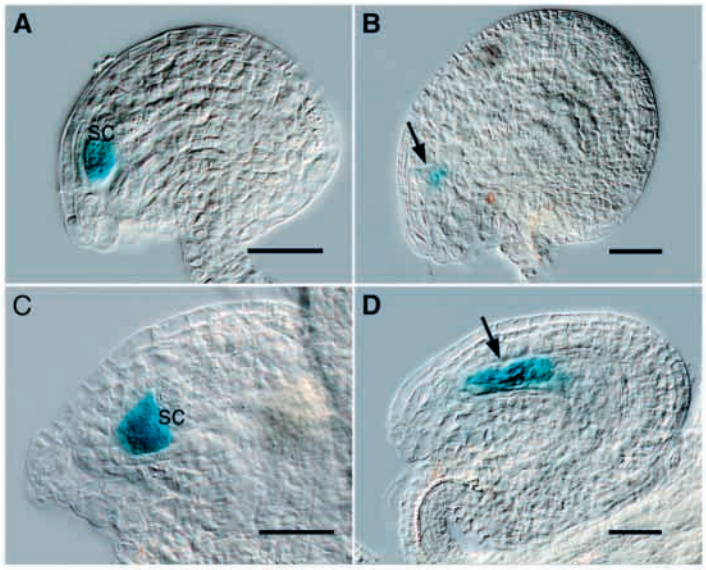


Fig. 5. Synergid-specific GUS activity of one out of three enhancer detector lines in wild-type and *feronia* ovules. (A) In mature wild-type ovules before fertilization, the GUS expression is restricted to the synergid cells (sc). (B) The GUS activity decreases significantly in wild-type ovules after fertilization (arrow). (C) In *feronia* (*fer*/+) ovules the synergid cells (sc) are stained before fertilization. (D) After PT invasion the GUS activity persists in the synergids (arrow) of mutant *feronia* embryo sacs. The invading PT appears to be covered by the GUS stain, indicating that it coils within the synergid. Scale bars: 30 μm.

the FG through one synergid and, if so, whether the synergid showed the typical signs of degeneration.

We first analyzed semi-thin sections of *feronia* and wild-type pistils stained with Toluidine Blue. We found that the coiling of the PT was restricted to the micropylar area of the embryo sac (Fig. 6A) and the PT did not usually enter the central cell (Fig. 6B). To investigate cytological changes within the synergid, we examined ultra-thin sections of wild-type and *feronia* siliques using transmission electron microscopy. In material prepared 6 HAP we found no signs of synergid degeneration, in either wild-type or *feronia* ovules (data not shown). Wild-type ovules collected 24 HAP had enlarged, the zygote had started to elongate and a few endosperm nuclei had formed (data not shown). The unfertilized ovules in the *feronia* mutant remained smaller and could easily be distinguished from fertilized ovules. In all analyzed mutant FG the micopylar area was filled with multi-layered membrane invaginations formed by the PT wall (Fig. 6C-F). The PT entered the embryo sac through or along the filiform apparatus, a specialized cell wall structure of the synergids (Fig. 6C,D). The receptive synergid was electron dense, contained an increased number of spherosomes, its nucleus was broken down and the organelles were disorganized. These structural modifications resembled the cellular changes previously documented for degenerated synergids in *Arabidopsis* (Mansfield and Briarty, 1991; Murgia et al., 1993). The PT often started to branch within the FG (Fig. 6E). In one case we observed the PT to bifurcate while entering the FG (Fig. 6C,D). The central cell and the egg cell appeared to be intact although the egg cell was often firmly clasped by the PT invaginations (Fig. 6F).

In conclusion, one synergid in *feronia* mutant FGs shows the

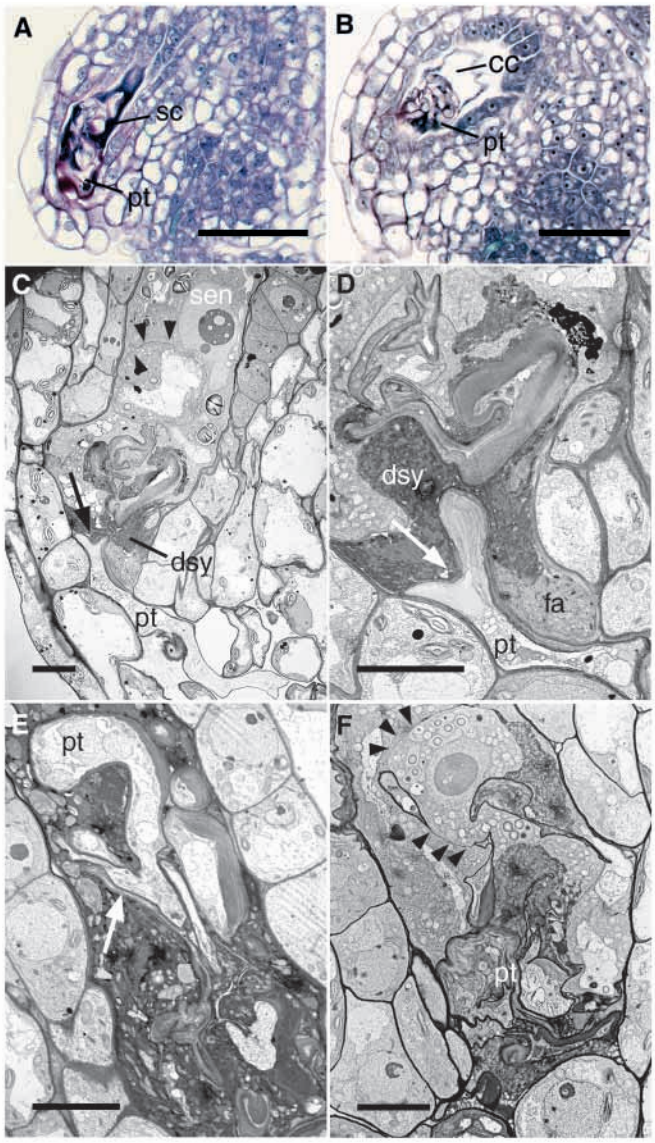


Fig. 6. Cytological analysis of pollen tube entry into *feronia* embryo sacs. (A,B) Semi-thin sections stained with Toluidine Blue. (C-F) Transmission electron micrographs of invaded *feronia* FG at 24 HAP. (A) The winding PT (pt) is covered by the dark staining remains of the synergid cell (sc). (B) PT invasion in mutant ovules is in most cases restricted to the synergid and does not enter the central cell (cc). (C) The PT enters the synergid and bifurcates (arrow) immediately after entry into the degenerated synergid (dsy). The egg cell membrane (arrowheads) appears to be intact and the secondary endosperm nucleus (sen) is present. (D) Adjacent but not successive section of the same sample as shown in C at higher magnification. The PT passes the filiform apparatus (fa) and enters the FG. Remains of the degenerated synergid surround the entering PT. (E) Branching (arrow) of the PT within the synergid. (F) The invading PT rearranges the micropylar area of the embryo sac, but the egg cell membrane appears to be intact (arrowheads). Scale bars: (A,B) 25 μm; (C-F) 5 μm.

typical cytological changes of synergid-specific degeneration. This synergid was the entry point for the PT, in accordance with observations of wild-type FGs. However, in *feronia* mutant FGs the PT starts to coil and branch within the synergid.

The electron dense remnants of the degenerated synergid always surrounded the PT invaginations suggesting that PT invasion is, in general, restricted to the synergid cell. Thus, the *feronia* mutation does not appear to disrupt the development and differentiation of the receptive synergid but rather a specific function required for gametophyte-gametophyte communication.

DISCUSSION

The female gametophytic mutant *feronia* controls pollen tube reception

The *feronia* mutant was isolated in a two-step screen designed to identify mutants with a defect in the reproductive function of the FG (Drews and Yadegari, 2002; Moore, 1997; Page and Grossniklaus, 2002). The mutant displayed a strongly reduced female transmission efficiency, indicating a predominant defect in the FG (Fig. 2B), but megagametogenesis and PT guidance were not affected. However, half of the FGs remained unfertilized, indicating a failure in the process of double fertilization.

After entering the synergid, the PT continued to grow, coiled and sometimes bifurcated (Fig. 3B,D; Fig. 6). Thus, the PT tip must be intact, because PTs elongate by polarized tip growth (Franklin-Tong, 1999; Hepler et al., 2001). Consequently, the PT must fail to rupture and discharge the sperm cells in *feronia* FGs, imparing double fertilization. The *feronia* mutant phenotype clearly shows that in *Arabidopsis* the FG participates actively in PT reception. The FG is known to control long-range guidance of the PT to the ovule (Higashiyama et al., 2001; Hülskamp et al., 1995b; Ray et al., 1997) and into the micropyle (Shimizu and Okada, 2000). These interactions are likely to involve the secretion of chemo-attractive compounds by the FG (Lush, 1999). In contrast, PT reception is achieved within the synergid by an immediate interaction of the PT with the FG. The defect in PT reception observed in the *feronia* mutant must, therefore, be the result of a deficient synergid function, i.e. a failure in the communication between the synergid and the PT.

Synergid specification and degeneration in *feronia* appear normal

The autolytic degeneration of one of the synergids is thought to be a prerequisite for PT rupture (Jensen and Fisher, 1968). An abnormal degeneration of the synergid could explain the defect in PT reception observed in the *feronia* mutant. However, based on our studies with synergid-specific markers, the synergids in *feronia* appear to be specified and differentiated normally (Fig. 5A,B). In wild-type plants, the GUS signal decreases in both synergids within 24 HAP, while the staining persists in *feronia* FGs. Taken together these data suggest that either synergid degeneration or normal pollen tube reception is required for the down-regulation of GUS marker gene activity. However, the investigation of the ultrastructure of mutant *feronia* FGs indicated that the receptive synergid shows the typical characteristics of degeneration (Fig. 6C-F). Both normal expression of synergid markers before PT entry and ultra-structural changes in the synergid after PT entry indicate that there is no basic defect in synergid development and differentiation in mutant *feronia* FGs. It is more likely that the persisting GUS activity after PT invasion is a consequence of unsuccessful PT reception in *feronia* FGs, as discussed below.

Synergid degeneration is not necessary for PT attraction as demonstrated by the *gfa*2 mutant in *Arabidopsis* in which the synergids fail to degenerate (Christensen et al., 2002). Despite normal PT guidance to the ovule, the PT does not enter the FG and mutant *gfa*2 embryo sacs remain unfertilized. In *feronia* the PT enters the FG by its normal route through the receptive synergid and the remains of the degenerated synergid cover the PT. Thus, the PT is exposed to the enzymatic environment that is responsible for the autolytic degeneration of the synergid. Therefore, in *Arabidopsis*, synergid degeneration might facilitate the entry of the PT into the embryo sac and possibly the targeting of the sperm cells to the female gametes, as suggested previously (Drews and Yadegari, 2002; Russell, 1992), but is not sufficient to accomplish PT rupture and sperm cell release.

The *feronia* mutation disrupts signalling between male and female gametophytes

The failure in PT reception in *feronia* is likely to be caused by the disruption of a signalling process between the male and female gametophyte. This view is consistent with the observed failure of PT reception in crosses between different rhododendron species. In these interspecific crosses, PT growth does not arrest and results in PT overgrowth in the embryo sac similar to the *feronia* phenotype (Kaul et al., 1986; Williams et al., 1986). This defect can be explained by the evolutionary divergence in specific, co-evolved recognition systems (Hogenboom, 1984) required to accomplish PT reception. The failure in PT reception observed in *feronia* mutants can be interpreted as the absence or change of a female gametophytic component involved in the direct communication between the female and the male gametophyte.

So far, it was unresolved whether the PT accomplishes growth arrest and PT rupture on its own or whether the FG, in particular the receptive synergid, actively participates in these processes. Investigations of PT reception in cotton (Jensen and Fisher, 1968) and spinach (Wilms, 1981) demonstrate that PT rupture is a controlled process. In cotton, sperm cell discharge is accomplished by a sub-terminal, and in spinach by a terminal, opening, which is sealed afterwards by a callose plug. If growth arrest and PT rupture are PT-intrinsic processes, then the PT requires a signal supplied by the FG to trigger these processes after entry into the FG. This signal might be absent in mutant FGs of *feronia*. But if arrest of growth and PT rupture need the active participation of the FG, then there are two possible interpretations for the observed phenotype in *feronia*: Either the mutant is defective in a novel recognition pathway enabling the FG to respond to the entering PT, or mutant FGs lack an essential component that either directly, e.g. by an enzymatic reaction, or indirectly, e.g. by inducing a signal transduction cascade, accomplishes PT growth arrest, rupture of the PT tip, and sperm cell release.

Other signalling events fail after pollen tube entry

It is known that PT reception immediately triggers several responses in the embryo sac. These include the movement of the egg nucleus (Faure et al., 2002), the targeting of the sperm cells to the female gametes (Huang et al., 1993; Huang and Sheridan, 1998), the degeneration of both synergids (Russell,

1996), and the loss of PT attraction by the FG. Mutant *feronia* FGs display a defect in at least two of these processes. First, the two synergids do not senesce normally, because we observed a persisting expression of synergid-specific gene expression in mutant FGs (Fig. 4D), and second, a distinct fraction of the mutant FGs attracts supernumerary PTs (Fig. 4).

The PT reaches the FG about 6-8 HAP in plants of the Columbia ecotype (Faure et al., 2002) but there will be some differences depending on ecotype and growth conditions. When we analyzed the expression of three independent synergid-specific molecular markers in wild-type plants, the GUS signal disappeared within 24 HAP in both synergids. As the half-life of GUS in the FG is rather short (12 hours after blocking protein biosynthesis with cycloheximide GUS activity is barely detectable; R. Baskar and U. G., unpublished data), the down-regulation in both synergids must be controlled by a general mechanism that is activated immediately after the PT enters the embryo sac. How this rapid response is accomplished remains unclear. In mutant *feronia* FGs GUS activity persists after PT invasion. Therefore, the normal signalling pathway leading to the repression of the GUS signals must be interrupted. One possibility is that mutant FGs do not respond to the entry of the PT, as discussed above. Alternatively, the global repression of gene activity in the synergids might be induced by components of the PT that are not discharged into mutant *feronia* FGs.

Whereas only one PT generally approaches the micropyle (Hülskamp et al., 1995b; Shimizu and Okada, 2000), we observed that ~10% of mutant *feronia* FGs attracted two or more PTs (Fig. 5). The PTs were able to enter the micropyle and invade the FG. How the FG prevents the arrival of supernumerary PTs is not understood (Lush, 1999; Smyth, 1997). Repulsive interactions between two PTs have been discussed as a possible mechanism (Lush, 1999; Shimizu and Okada, 2000). But the observation that two or more PTs can enter mutant FGs in *feronia* mutants argues against such a repulsive interaction. Another explanation is an immediate loss of PT attraction after functional PT reception, provided that simultaneous growth of PTs to the same FG is prevented – either because the amount of the chemo-attractant is very limited or is immediately sequestered by the approaching PT. Mutant *feronia* FGs do not repress synergid-specific GUS markers, indicating that a failure in PT reception does not induce a global repression of gene activity in the synergids. Therefore, it is likely that the expression of a synergid-borne attractant persists as well and results in the attraction of supernumerary PTs.

The characterization of the female gametophytic mutant *feronia* demonstrates that the FG controls PT behaviour after entry into the synergid. Because there is no developmental defect in mutant *feronia* FGs and the synergids undergo normal differentiation and degeneration, the *feronia* mutation must affect an, as yet unidentified, active communication process between PT and the synergid. The isolation of the *FERONIA* gene will provide more information about the complex interactions between the male and female gametophyte necessary to accomplish the process of double fertilization in angiosperms.

Note added in proof

While our paper was in press, the description of *sirene*, a mutant with a similar phenotype, was published (Rotman et al., 2003).

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