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RESEARCH PAPER

Sporophytic control of pollen tube growth and guidance in maize

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Abstract

Pollen tube germination, growth, and guidance (progamic phase) culminating in sperm discharge is a multi-stage process including complex interactions between the male gametophyte as well as sporophytic tissues and the female gametophyte (embryo sac), respectively. Inter- and intra-specific crossing barriers in maize and *Tripsacum* have been studied and a precise description of progamic pollen tube development in maize is reported here. It was found that pollen germination and initial tube growth are rather unspecific, but an early, first crossing barrier was detected before arrival at the transmitting tract. Pollination of maize silks with *Tripsacum* pollen and incompatible pollination of *Ga1s*/*Ga1s*-maize silks with *ga1*-maize pollen revealed another two incompatibility barriers, namely transmitting tract mistargeting and insufficient growth support. Attraction and growth support by the transmitting tract seem to play key roles for progamic pollen tube growth. After leaving transmitting tracts, pollen tubes have to navigate across the ovule in the ovular cavity. Pollination of an embryo sac-less maize RNAi-line allowed the role of the female gametophyte for pollen tube guidance to be determined in maize. It was found that female gametophyte controlled guidance is restricted to a small region around the micropyle, approximately 50–100 μm in diameter. This area is comparable to the area of influence of previously described ZmEA1-based short-range female gametophyte signalling. In conclusion, the progamic phase is almost completely under sporophytic control in maize.

Key words: Female gametophyte, maize, pollen tube guidance, prezygotic barriers, transmitting tract, *Tripsacum*.

Introduction

Wide hybridization between different taxonomical plant species is very common in nature. In some genera like *Quercus*, hybrids can be more abundant in the landscape of an area than the pure parental species and form stable hybrid swarms (Whittemore and Schaal, 1991). Moreover, hybridization barriers between plants of the same or related species is thought to be one of the driving forces of speciation and therefore represents an important process in flowering plant evolution (Rieseberg and Willis, 2007). For plant breeders, hybridization of crop plants with closely related wild species is an important way of introducing new traits, such as biotic and abiotic resistance, into economically important species. Unfortunately, hybridization often has drawbacks in terms of reduced seed set and fertility of the F_1 generation. Understanding the mechanism of pre- and post-zygotic inter- and intra-specific crossing barriers in crop plants are therefore of great interest for reproductive and evolutionary plant biology as well as for plant breeding. In particular, the long studied prezygotic barriers related to pollen tube (PT) germination, growth, and guidance represent the major hybridization controls in nature (Arnold and Hodges, 1995; Widmer *et al.*, 2009).

Various stages of progamic PT development and growth from pollen–stigma contact to sperm cell discharge offers many possibilities to reject alien pollen and to prevent unfavourable fertilization by alien pollen. In the model plant system *Arabidopsis thaliana* Heynh., for example, progamic male gametophyte development has been divided into five distinct phases (Johnson and Preuss, 2002; Swanson *et al.*, 2004). Phases I–III, from capture of the pollen grain at the stigma surface, tube growth initiation, and navigation between the stigma cells towards and inside the transmitting

tract (TT), are mainly governed by anatomical aspects of the stigma and style as well as species-specific pollen–stigma interaction and signalling. In Phase IV, during growth along the ovule surface, PTs are thought to depend on signals from both the haploid female generation and the diploid maternal tissue of the ovule. In Phase V, the micropyle is targeted and the sperm cells are released inside the egg apparatus. This final phase is thought to be under gametophytic control (Shimizu and Okada, 2000; Johnson and Preuss, 2002; Higashiyama and Hamamura, 2008). Similar determinations of PT growth phases have been made for other eudicots such as cotton (*Gossypium barbadense* L.; Ram *et al.*, 2008) and *Torenia fournieri* Lind. (Kikuchi *et al.*, 2007). Due to anatomical differences related to a single ovule inside the ovary, the progamic male gametophyte development in grasses (Poaceae) has been classified in a slightly different way (Heslop-Harrison, 1982). Phases I–III in maize (*Zea mays* L.), for example, can be regarded as homologous to those described in *A. thaliana*. However, during Phase IV, the TT in the upper ovary wall (upper style) plays an important role in PT number reduction and is the last location where the PTs are growing between sporophytic cell layers. At the end of the TT, PTs leave the sporophytic tissue to enter the ovarial cavity and to grow at the surface of the inner integument towards the micropylar region (Heslop-Harrison *et al.*, 1985).

The ovarial cavity and the micropyle are thought to contain chemotropic signals to guide the growing PT (Heslop-Harrison, 1982; Márton *et al.*, 2005; Higashiyama and Hamamura, 2008; Okuda *et al.*, 2009). In the last decade, many small proteins and other general compounds like calcium, γ-amino butyric acid, or nitric oxide have been discussed to be involved in progamic PT germination, adhesion, growth, and guidance. Most advances have been made in the Brassicaceae and Solanaceae families including their well understood self-incompatibility (SI) systems (for reviews see Hiscock and McInnis, 2003; Swanson *et al.*, 2004; McClure and Franklin-Tong, 2006). SI is a first distinct obstacle for the male gametophyte, preventing not only self- but also hetero-pollination by alien species if severe physiological and anatomical disharmony between pollen and female organs is neglected. Hybridization between closely related self-compatible and self-incompatible plant species regularly fail, if the self-incompatible species is pollinated with pollen from the self-compatible one. However, hybridization can be successful if pollination is carried out vice versa (Lewis and Crowe, 1958). In contrast to the Brassicaceae and Solanaceae families, much less is known about SI in the Gramineae. Grasses are known to have two loci (S and Z) based on a gametophytic SI-system. Although the grass SI-system is genetically gametophytic, the appearance is sometimes more similar to the sporophytic SI system of the Brassicaceae. Pollen can be rejected at the level of pollen hydration and germination but also during growth through the style and even at the ovule surface or via post-fertilization events (Yang *et al.*, 2008). In the important crop plant *Z. mays*, which is generally self-compatible, several loci are known to lead to incompatible pollination or to influence progamic male gametophyte development. Among these, the *gametophte factors* (*ga*) are best described. When silks, homozygous for the dominant *Ga1* allele, for example, are pollinated with a mixture of *Ga1* and *ga1* pollen, those carrying the recessive allele are rarely involved in fertilization (Nelson, 1994). The strongest phenotype is observed in the pollination of *Ga1s*/*Ga1s*-silks with *ga1*-pollen. This combination leads to a complete lack of seed set due to a slowed and finally interrupted growth of PTs in the silks (House and Nelson, 1958; Bedinger and Fowler, 2009). Another genetically linked, but distinct crossing barrier is based on the *teosinte crossing barrier 1* (*tcb1*) locus mediating unilateral crossing barrier between maize and its closely related subspecies teosinte. Maize pollen, usually carrying the recessive *tcb1* allele, is unable to fertilize teosinte (generally homozygous for the *Tcb1s* allele). Both barriers act independently and through incongruity rather than active rejection. In both cases, *ga1* and *tcb1*, the genotype affects the male gametophyte and sporophytic maternal tissues. The recessive alleles are thought to be null alleles (Kermicle and Evans, 2005). Hitherto, the molecular basis of both phenomenon remained unknown. Other maize mutants like *white pollen1* (*whp1*) and *colorless2* (*c2*), both defective in chalcone synthase and thus flavonol biosynthesis, are also hampered in PT growth in the style (Pollak *et al.*, 1995). A member of a sister genus of *Zea*, namely *T. dactyloides*, has been shown to be able to hybridize with maize at unnatural conditions. Whereas pollination of *Tripsacum* with maize is regularly successful, *T. dactyloides* pollen in general is only able to fertilize maize if silks are cut back to a length of less than 2.5 cm (Mangelsdorf and Reeves, 1931).

Finally, in analogy to Phase V of progamic male gametophyte development in *A. thaliana* and other dicotyledonous species, species-specific signals or barriers by the female gametophyte (embryo sac) are postulated also to exist in grasses controlling PT growth and guidance around the micropyle. A small secreted protein, ZmEA1, expressed in the egg apparatus, is the first candidate for a micropylar guidance signal in maize (Márton *et al.*, 2005), and recently defensin-like proteins secreted by the synergids have been shown to guide the PT towards the egg apparatus in the micropylar region of *T. fournieri* in a species-specific manner (Okuda *et al.*, 2009).

In order to gain more insights into crossing barriers and progamic PT development in maize at the cellular level, cross-pollination experiments were performed among and between maize and *T. dactyloides*, and in addition PT behaviour of other plant species on maize and *T. dactyloides* silks was analysed. Pollen germination efficiencies and tube growth in silks as well as in the ovarial cavity were investigated and compared, including also the genetic *gametophyte factor Ga1s*/*ga1*-system. To determine the role of the female gametophyte for PT attraction during progamic Phases IV and V, a novel mutant line was applied, displaying fully differentiated maternal ovary tissues but completely disintegrated embryo sacs.

Materials and methods

Plant material

In addition to wild-type maize inbred lines A188 and K55, near isogenic lines *Ga1s*/*Ga1s*, *Ga1s*/*ga1* and *ga1*/*ga1*, all based on the genetic background of K55, and the respective backcrosses were used. Tetraploid and hexaploid accessions of *T. dactyloides* were applied for various experiments. Rice (*Oryza sativa* L. ssp. *japonica*) pollen was collected from the commercial inbred line M 202, and from greenhouse-grown grasses *Poa nemoralis* L. and *Lolium multiflorum* Lam. All Poaceae were kept in the greenhouse under long-day conditions (16 h of light). The temperature was kept at 25 °C during the light period and at 18 °C in the dark. Flowering of rice was induced by short-day treatment (9 h of light) for 2 weeks. *A. thaliana* (ecotype Columbia-O) was raised under short-day conditions and shifted to long-day conditions after 4 weeks. Long- and short-day chambers were kept at 20–22 °C and 70% humidity. Lily (*Lilium longiflorum* Thunb.) flowers were obtained as cut material from local flower shops. Pollen from all plants except maize and *T. dactyloides* was obtained by harvesting anthers 1 d before or at anthesis. Maize and *T. dactyloides* pollen was obtained by shaking fresh pollen into a paper bag between 09.30 and 10.00 a.m. Older pollen was removed from tassels by vigorous shaking the evening before pollen harvest.

Pollination and sample preparation

Pollinations were carried out either *in vitro* or in the greenhouse using whole plants. For *in vitro* pollination, emasculated flowers were placed into a humid chamber and pollen was applied by shedding or by using a fine brush. In order to prevent unintended pollination of distal parts of the silks, they were covered with a piece of paper. After various incubation times, silks were fixed for aniline blue staining as described below. For cross-sections, fixed and stained silks of maize and *T. dactyloides* were imbedded in 5% low melting agarose. Slices of 80 μm were cut using a vibratome (Leica VT 1000S) and observed under an inverted microscope (Nikon Eclipse 1500). *In vitro* pollinated silks were incubated in a humid chamber for 3–24 h at 21 °C in the dark. Seed set after pollination of plants was monitored after 2 weeks.

Aniline blue staining (modified after Martin, 1959)

Silk tissue and ovules were fixed overnight in 9:1 v/v ethanol:acetic acid at 4 °C. Fixed samples were rehydrated by an ethanol series (70%, 50%, and 30%) each for 5 min and washed with 0.1 M potassium phosphate buffer pH 8.0. Subsequently, samples were incubated for 5 min in 10% chloral hydrate and afterwards for 10 min in 5 M sodium hydroxide solutions each at 65 °C. After each step, samples were washed with potassium phosphate buffer. The cleared and smoothed tissue was then stained for 15 min at 21 °C or for up to several days at 4 °C with 0.1% aniline blue solution (water blue, Fluka) prepared with potassium phosphate buffer (see above). Specimens were mounted with fresh staining solution on a slide with a cover slip and analysed on an inverted microscope (Nikon Eclipse 1500) with near UV excitation.

Histological studies of fixed and cleared ovules

Whole cobs were treated according to a fixing/clearing method using Kasten's fluorescent periodic acid–Schiff's reagent described by Vollbrecht and Hake (1995). The phases for hydration and dehydration of ears were prolonged from 20–30 min in each step and ears were dissected after clearing with methyl salicylate (Young *et al.*, 1979). Samples were mounted in methyl salicylate on glass slides under a cover slip and analysed with a LSM510-META confocal laser scanning microscop (Zeiss) with 488 nm excitation and a LP 505 filter.

Results

Pollen germination efficiency

In an initial experiment, the ability of pollen from different plant species to germinate on silks of maize and *T. dactyloides*, respectively, was determined. In order to rule out bias of germination rates by inadequate temperature and air humidity conditions, silks or stigmas of donor plants were always *in vitro*-pollinated in the same growth chamber. Subsequently, pollinated silks were stained with aniline blue to determine the ability of PTs to penetrate the stylar tissue (Fig. 1A–H). With the exception of lily, all PTs studied were able to germinate, but with the exception of maize and *T. dactyloides* either failed to invade or stopped growth within the silk hairs. Germinated pollen of *A. thaliana* is shown as an example in Fig. 1D and H. Only the large PTs of maize (Fig. 1A, B, G) and *T. dactyloides* (Fig. 1C, E, F) were able to grow completely through the silk hairs and reach the stigmatic tissues harbouring the TTs. Due to this finding, further detailed *in vitro* experiments were carried out only with pollen of these two plant species. To normalize the germination rates obtained, the average of germinated pollen on maize or *T. dactyloides* silks was determined in relation to the average of germination rates observed after self-pollination (detailed numbers are given in Supplementary Table S1 at *JXB* online). These percentages are shown in Fig. 1I for maize silks and in Fig. 1J for *T. dactyloides* silks. Notably, *T. dactyloides* pollen show high relative germination efficiencies on both self and alien species, whereas germination efficiency of maize pollen is reduced to 50% on *T. dactyloides* silks. Pollen of other, more distantly related Poaceae showed relative germination efficiencies of 30–60% on silks of both maize and *T. dactyloides*, respectively. Interestingly, the dicotyledonous plant *A. thaliana* shows germination efficiencies in the range of distantly related Poaceae, while pollen of the monocot *L. longiflorum* did not germinate on grass silks.

Pollen tube growth range in maize and Tripsacum dactyloides silks

Pollen tube growth length is known to be the critical factor in the unilateral incompatibility phenomenon between *ga1*-pollen and *Ga1s*/*Ga1s*-silks (House and Nelson, 1958). The ability of *T. dactyloides* pollen to fertilize maize when silks are cut back suggests that this might also be the case for the natural maize–*Tripsacum* crossing barrier (Mangelsdorf and Reeves, 1931). In order to compare both hybridization barriers and tube growth range, silks, which represents stigmatas, of the maize inbred lines A188 and K55 as well as the near isogenic line *Ga1s*/*Ga1s* and its backcross progenies with K55 were analysed over a length of at least 10 cm. Cob segments were placed in a humid chamber, pollinated, and analysed as shown in Fig. 2A. The presence of PTs in the various silk segments was monitored by aniline blue staining (Fig. 2B–G). After pollination of silks from A188 with hexaploid and tetraploid *T. dactyloides* pollen, PTs were frequently found in the segments 0–4 cm

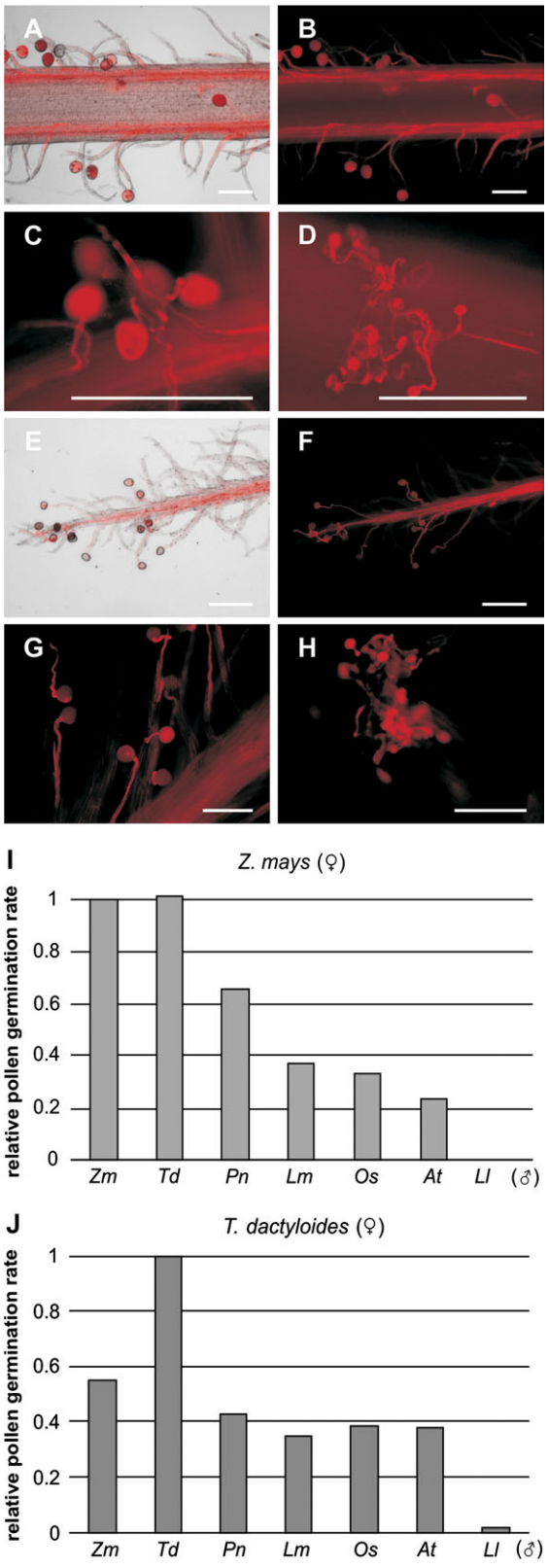

Fig. 1. Germination rates of pollen from various monocot and dicot species on silks of maize and *Tripsacum dactyloides*, respectively. Silks of maize (A–D) and *Tripsacum dactyloides* (E–H) were pollinated with pollen from different plant species and incubated in a humid chamber at room temperature. All specimen were stained with aniline blue and false red colour animated for better contrast. Germination of maize pollen grains is shown in (A, B) and (G), that of *T. dactyloides* pollen in (C) and (E, F), while *Arabidopsis thaliana* pollen are shown in (D) and (H). To determine the relative pollen germination efficiency (I, J), germination was evaluated after 3–7 h without staining and each value related to the germination rate after self-pollination. See Supplementary Table S1 at *JXB* online for details. Abbreviations: *Zm, Zea mays; Td, Tripsacum dactyloides; Pn, Poa nemoralis; Lm, Lolium multiflorum; Os, Oryza sativa; At, Arabidopsis thaliana; Ll, Lilium longiflorum.* Scale bars: 200 μm.

distal to the pollination area. In rare cases, PTs were found in segments 4–8 cm distal but never in the proximal (>8 cm distal) part of the silk (Fig. 2H). Many *T. dactyloides* PTs grew outside the TT (Fig. 2F). With few exceptions, when maize PTs outside the TT stopped growth after a few cm (Fig. 2G), close to 100% of maize PTs from inbred line A188 grew straight inside the TT (Fig. 2D, E) and continued growth all the way through the silk tissues. Pollination of *Ga1s*/*Ga1s*-silks with pollen from different maize genotypes and *T. dactyloides* revealed that, in the incompatible *Ga1s*/*Ga1s*×*ga1*/*ga1* crossing, PTs display similar growth behaviour to that in maize×*T. dactyloides* pollination experiments (Fig. 3A). *ga1*-PTs stop their growth more frequently and after a shorter distance than *T. dactyloides* PTs. *T. dactyloides* and *ga1* PTs reach the proximal part of *Ga1s*/*Ga1s* silks in very rare cases. In this experiment, no striking difference was found between PTs originated from *Ga1s*/*Ga1s*- and *Ga1s*/*ga1*-plants, respectively. Silks of heterozygous *Ga1s*/*ga1*-plants show intermediate PT growth length behaviour when pollinated with *ga1*-pollen but not when pollinated with *T. dactyloides* (Fig. 3B). On silks of the inbred line K55, PT growth of all maize genotypes and *T. dactyloides* show the same behaviour to that on silks of the inbred line A188 (Fig. 3C). In order to address the question of the physiological and cellular basis of reduced PT growth length, cross-sections of maize silks at 2 cm and 6 cm distal to the area of pollination were investigated in order to study the location of PTs inside the style. Firstly, the position of the vascular tissue was determined by safranin/astra-blue or phloroglucin/HCl staining in unpollinated silks (data not shown) or after aniline blue staining (Fig. 4A). Notably, tracheae vessels of maize stained both for safranin/phloroglucin (cellulose) and aniline blue (callose). The latter was used as a marker to visualize tracheae vessels in pollinated silks. In control pollinations of maize (Fig. 4B) and *T. dactyloides* (Fig. 4G) silks with self-pollen, it was shown that PTs were detected almost exclusively in the intercellular spaces between TT cells, which are in close association with the vascular bundles (Fig 4A, F). In the incompatible pollination of silks of the genotype *Ga1s*/*Ga1s* with recessive pollen (*ga1*), most of the PTs were found inside the TTs. Few PTs failed to target the TT and grew below the epidermal cell layer towards the ovule (Fig. 4C). These PTs showed shorter growth length than those growing inside the TT. When *T. dactyloides* pollen was applied on maize silks, many PTs failed to enter the TT (Fig. 4D) and instead displayed

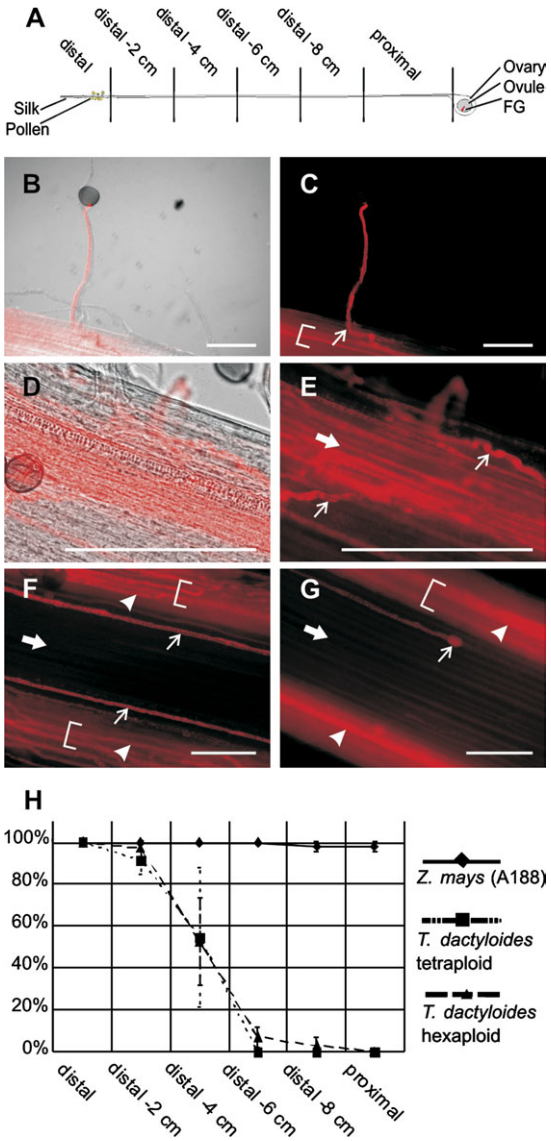

Fig. 2. Pollen tube growth range in silks of maize inbreed line A188. (A) Cob segments have been placed in a humid chamber and were pollinated with pollen from maize as well as tetraploid and hexaploid *Tripsacum dactyloides*, respectively. 16 h after incubation at room temperature, silks were cut in 2 cm pieces with proximal ends varying in length. Specimens were fixed, analysed after aniline blue staining and false red colour animated for better contrast (B–G). (B, C) Maize pollen tubes outside and inside the silk tissue (arrow in C) within the transmitting tract (bracket). (D, E) Pollen tubes of both species, maize and *T. dactyloides* grow towards the transmitting tracts surrounding the parenchymal cells (arrow in E). (F) Some *T. dactyloides* pollen tubes grew outside the transmitting tract (arrows) and (G) arrested after shorter growth than those inside the transmitting tracts (brackets; pollen tubes inside the TT are marked with arrowheads). *T. dactyloides* pollen tube (arrow) growth arrest was observed in silk segments at 2–8 cm distance from the area of pollination. Pollen tubes inside transmitting tracts (brackets; pollen tubes marked with arrowheads) continued growth. (H) Silk segments containing pollen tubes were counted and set in relation to the total number of silks investigated. Brackets indicate transmitting tracts. Bold arrows indicate pollen tube growth direction towards the ovary. Scale bars: 200 μm.

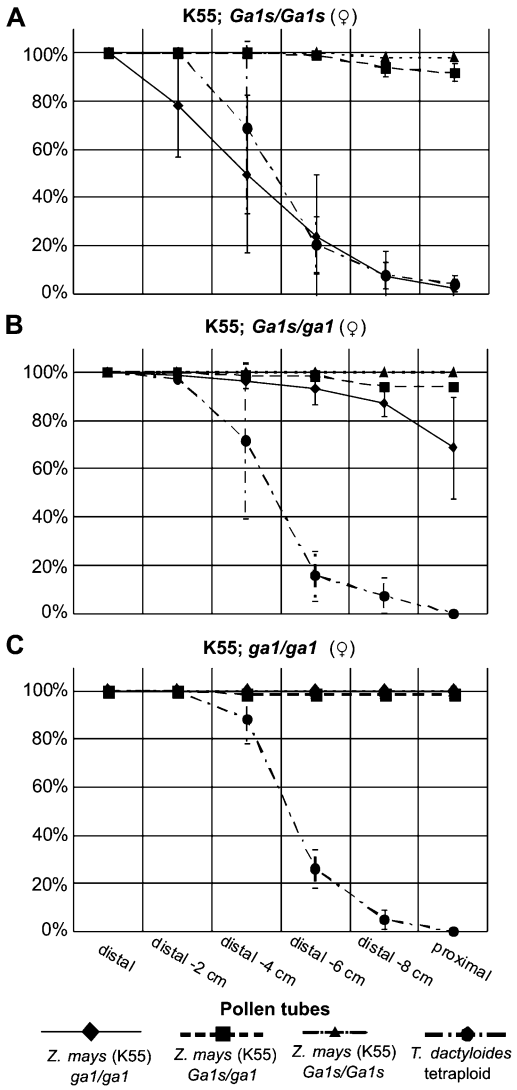

Fig. 3. Pollen tubes growth range in silks of maize inbred line K55 harbouring different allele combinations of *Ga1s*. Cob segments were placed in a humid chamber and were pollinated as indicated. After 16 h incubation at room temperature, silks were cut, stained and pollen tubes counted as described before. (A) Silks dominant for *Ga1s*. (B) Silks heterozygous for *Ga1s*. (C) Silks recessive for *Ga1s* (*ga1/ga1*).

a rather homogenous distribution throughout the silk tissue. Those *T. dactyloides* PTs that found their way inside the TT achieved a longer growth length, but finally growth was arrested, on average after 2–4 cm (Fig. 4E). Cross-sections of *T. dactyloides* silks (Fig. 4F–H) revealed that all PTs of both *T. dactyloides* (Fig. 4G) and maize (Fig. 4H) quickly reach the TTs and make their way towards the ovary harbouring the single ovule.

Pollen tube guidance towards the ovular cavity and micropyle

PT guidance in the ovular cavity is thought to be controlled by specific chemotropic signals secreted by the female gametophyte. In maize plants, where the *ZmEA1* is down

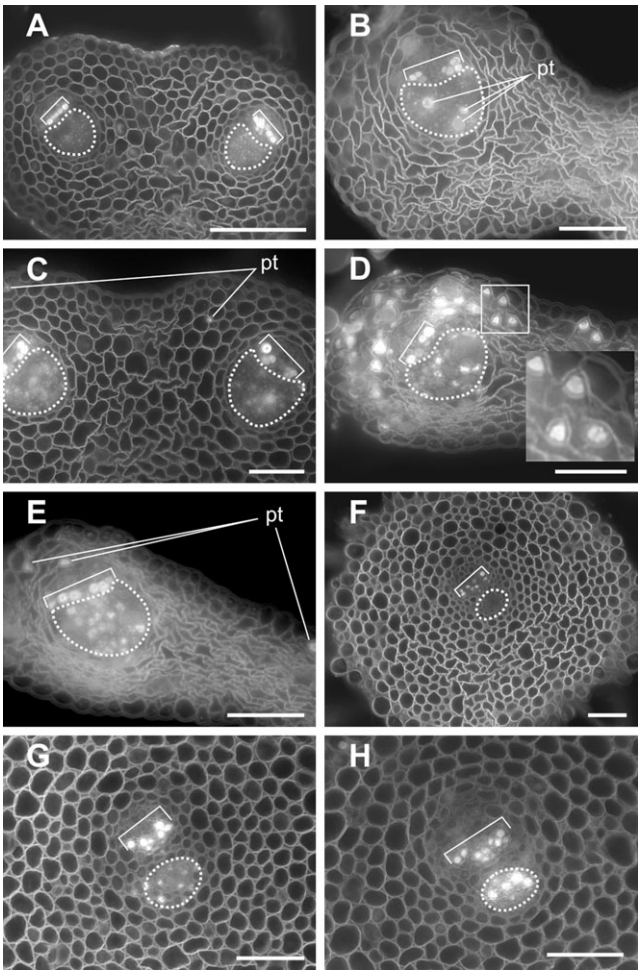

Fig. 4. Cross-sections of maize and *T. dactyloides* silks stained with aniline blue. (A) In an unpollinated maize silk, xylem elements (brackets) of the two vascular bundles show strong aniline blue staining whereas other cell walls lead to a light background signal. The transmitting tract (encircled) is composed of small longitudinal cells in close proximity to the xylem elements. (B) 24 h after pollination with wild-type maize pollen, pollen tubes appear as large, round, and brightly stained structures growing in the intercellular space between TT cells (only one vascular bundle is visible). (C) After pollination of *Ga1s*/*Ga1s* silks with recessive pollen (*ga1)*, some pollen tubes (pt) grew outside of the TT towards the ovule. (D) The same phenomenon can be seen regularly in maize silks pollinated with *T. dactyloides* pollen. If pollinated silks are cut at the site of pollination, several pollen tubes grew outside the TT right below the epidermis (four pollen tubes between parechymal cells are visible in the onset). (E) These pollen tubes stop their growth earlier and are therefore less abundant in more distal parts of the silk. (F) Compared to maize, *T. dactyloides* transmitting tracts are more deeply embedded into the silk tissue and contain only one vascular bundle (bracket) with associated TT (encircled). Cross-section of pollinated silks show pollen tube growth exclusively in TT in *T. dactyloides* silks pollinated either with *T. dactyloides* (G) or maize (H) pollen. Scale bars: 50 μm.

regulated by RNAi, PTs grew in close proximity to the micropyle, but failed to enter it for successful fertilization (Márton *et al.*, 2005). In order to study the role of the female gametophyte for ovular and micropylar guidance in maize, novel RNAi-lines lacking functional embryo sacs were used. Knock down of the diSUMO-like protein gene *ZmDSUL* of maize results in an embryo sac development arrest at stage FG5/6 followed by complete disintegration of the female gametophyte without affecting the maturation of the embryo sac (Srilunchang *et al.*, 2010). Mature ovules of these mutants contain fully differentiated sporophytic ovule tissues, but completely disintegrated embryo sacs (Fig. 5B). These RNAi-lines enabled us to investigate the role of the female gametophyte for PT guidance. Using wild-type ovules, PTs grew inside the ovular cavity after leaving the two TTs. PTs continued growth towards the micropyle and one PT penetrates the intercellular spaces between the micropylar nucellus cells to achieve double fertilization (Fig. 5C, D). By contrast, PTs grew at the surface of mutant ovules towards the micropyle until an area of approximately 100 μm from the centre of the micropylar cone (Fig. 5E, F). Arriving PTs of maize or *T. dactyloides* showed the same behaviour and seem either not to be attracted or repelled from the micropyle. Interestingly, late coming PTs are also excluded from an area of approximately the same diameter on wild-type ovules (Fig. 5C, D). These experiments indicate that, with the exception of micropylar short-range guidance, PT guidance in maize is completely controlled by the tissues of the sporophyte.

In order to address the fact that in incompatible pollinations some PTs are still able to reach and fertilize the embryo sac, mass pollinations of maize and *T. dactyloides* plants with pollen of various grasses were carried out (Table 1). First, it was possible to confirm the findings of incompatibility reactions after pollinating maize plants with full-length silks. Silks of each cob were separated into two populations and pollinated either at one side always with pollen from A188 or on the other side with alien or incompatible pollen (see Supplementary Fig. S1A at *JXB* online). It was found that the existing barriers in the maize×*T. dactyloides* and the *Ga1s*/*Ga1s*×*ga1*/*ga1* crossings can be overcome by shortening maize silks to a length of less than 5 cm and by applying pollen directly to the huskless cob (see Supplementary Fig. S1B at *JXB* online). Embryo development and seed set was only observed after silk shortening. Pollination of tetraploid *T. dactyloides* with pollen of various Poaceae species led to a significant number of developed seeds (Table 1). Besides undeveloped (10–40%) and fully developed seeds (10–50%), a number of seeds were obtained (10–40%) which were aborted (see Supplementary Fig. S1C at *JXB* online).

Discussion

Maize is generally considered as a self-compatible species (Yang *et al.*, 2008), but many popcorn strains cannot be fertilized by pollen of dent, sweet, and flints strains, although the reciprocal crosses are successful (House and Nelson, 1958, Kermicle and Evans, 2005). In particular, the dominant gametophytic factor *Ga1-s* present in many

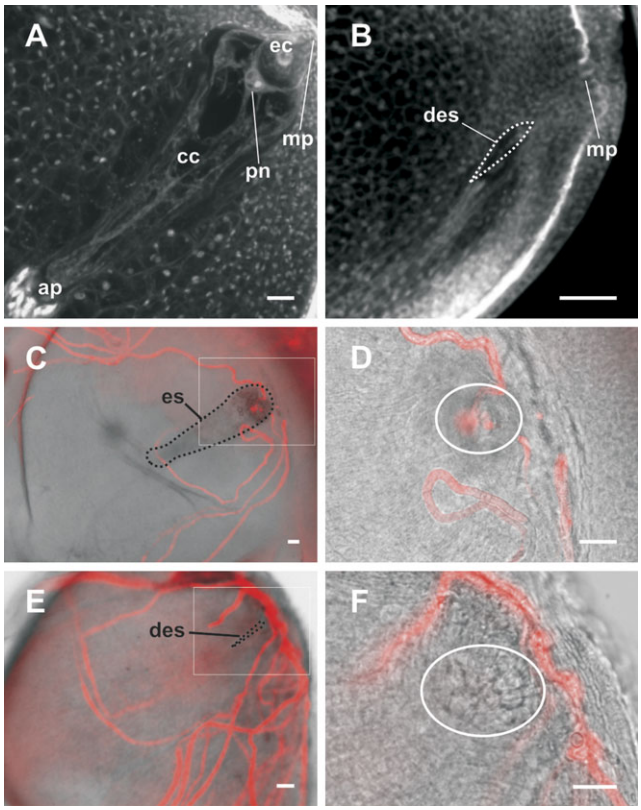

Fig. 5. Pollen tube growth and attraction in the micropylar region of the ovule. (A) CLSM longitudinal section through a WT ovule displaying a mature female gametophyte (FG). (B) *fg*-RNAi mutant ovule lacking a functional FG that is completely disintegrated after stage FG5 (Srilunchang *et al.*, 2010). (C) WT ovule 24 h after pollination and aniline blue staining (false colour red staining was used for better visibility of pollen tubes). Several pollen tubes arrived at the ovule surface and grew towards the micropylar region. One pollen tube grew inside the micropyle and released its contents inside the receptive synergid. The female gametophyte is encircled. The inset indicates the enlarged region shown in (D). (D) Only one pollen tube succeeded in entering the micropyle (circle) and additional pollen tubes seem to be no longer attracted by the micropylar region. (E, F) Ovules of the *fg*-RNAi mutant line: the female gametophyte is disintegrated (encircled) and pollen tubes (arrowheads) grew in 50–100 μm proximity to the centre of the micropylar cone (circle), but did not enter. Abbreviations: ap, antipodal cells; cc, central cell; des, degenerated embryo sac; ec, egg cell; es, embryo sac; mp, micropyle; pn, polar nuclei. Scale bars: 50 μm.

varieties of popcorn is involved in the prevention of fertilization (Nelson, 1952). The molecular nature of Ga1-s is not known to date. After applying alien pollen on maize and *T. dactyloides* silks, our investigations revealed the occurrence of inter-specific crossing barriers at various levels in maize. With the exception of lily, pollen capture, hydration, and germination do not seem to represent essential crossing barriers. In general, a striking physiological difference between the plant species analysed is the separation of plants into 'dry'- and 'wet'-stigma types.

Table 1. Seed set after pollination of maize and *T. dactyloides* silks with pollen from various grass species

Female	Male	Sum silks	Seed set	Efficiency
Z. mays silks	*Z. mays*	1768	690	37%
>10 cm	*T. dactyloides*	461	0	0%
	L. multiflorum	344	0	0%
	O. sativa	289	0	0%
	P. nemoralis	319	0	0%
Z. mays silks	*Z. mays*	894	289	32%
<5 cm	*T. dactyloides*	742	274	36%
	O. sativa	217	0	0%
T. dactyloides	*T. dactyloides*	96	48	50%
	Z. mays	36	8	22%
	L. multiflorum	24	3	13%
	O. sativa	21	4	19%
	P. nemoralis	20	2	10%
	No pollen	27	0	0%

Among the plant species used in this study, only lily is of the 'wet' stigma type, while grasses and *Arabidopsis thaliana* belong to the 'dry'-stigma type (Heslop-Harrison and Shivanna, 1977; Swanson *et al.*, 2004) explaining the incompatibility of lily pollen to germinate either on maize or on *T. dactyloides* silks. Pollen adhesion at the silks is also known as a critical factor for successful pollination (Bedinger and Fowler, 2009). Our observations indicate that lack of adhesion does not represent a hurdle in maize and *T. dactyloides*, but most PTs were unable to enter the silk hairs, which thus represents a first hybridization barrier of pollen from most plant species in maize and *T. dactyloides*. This finding further supports the assumption that specific factors from the PT and silk hair, like the xylanase XYN1 or other hydrolases, might enable the PT to invade the silk hairs (Bedinger and Fowler, 2009). A bias in these interactions prevents pollination of maize and *T. dactyloides* by distantly related plant species, but not by pollen from the sister genus. This barrier can thus be classified as an 'early growth arrest' of PTs.

Further obstacles are connected to the TT and lead to reduced PT growth length. PT growth length reduction in maize was first described as a unilateral crossing barrier caused by the *Ga1s*-allele (Nelson, 1952; House and Nelson, 1958). Although not studied at the cellular level, it has been reported that PT growth length is crucial to overcome crossing barriers existing between teosinte and maize (Kermicle and Evans, 2005) as well as teosinte and *T. dactyloides* (Mangelsdorf and Reeves, 1931). In our experiments, it was found that PT growth length in the incompatible *Ga1s*/*ga1* system is comparable to that of the growth length of *T. dactyloides* PTs in maize silks. House and Nelson (1958) reported a growth arrest of *ga1*-pollen after 2–3 cm, while compatible pollen displayed a linear growth of about 12 mm h^{-1}. Calculations indicated that maize pollen grains could support around 2 cm of tube growth using exclusively endogenous reserves (Heslop-Harrison, 1982; Heslop-Harrison *et al.*, 1984). This finding suggests that lack of further growth support by the

sporophytic tissues might represent the major cause of tube arrest described above. In both cases it was found that PTs are less precisely targeted to the TT. The majority of mistargeted PTs were observed in the silk tissue right below the epidermal cell layer. However, even PTs growing inside the TT tissue stopped growth after an additional 1–2 cm compared with the PTs growing outside the TT. The observation that the smaller *T. dactyloides* pollen can form tubes longer than 2 cm, especially inside the TT, indicates that its PTs are principally capable of taking up nutrients from the alien host silk. Under natural conditions, targeting to the TT is a prerequisite of PTs in order to grow through the maize silks completely. Our data thus supports an old hypothesis from Heslop-Harrison *et al.* (1985) that TT cells attract PTs by the secretion of chemotropic signals. This process is hampered in the *Ga1s*/*ga1* and the maize–*T. dactyloides* barriers suggesting that the signalling molecules involved might be species-specific. PT mistargeting of the TT occurs less frequently in an incompatible *Ga1s*/*ga1* crossing compared with maize–*Tripsacum* crossings, which indicates that both barriers might be based on the same physiological process, but are impaired to a different extent. Both barriers do not appear in the reciprocal crossings, because PT growth-defects or TT-mistargeting was not observed. Nutrition problems similar to *colourless2* (*c2*) or *white pollen1* (*whp1*) mutants could explain our findings concerning *T. dactyloides* PT growth arrest in maize silks. Both mutants are self-incompatible but can be rescued either by shortening silks (Pollak *et al.*, 1995) or by mixing pollen with flavonols (Mo *et al.*, 1992). If flavonols were not supplemented, PTs grew to a length of only 2.5–4 cm (Pollak *et al.*, 1995).

When PTs leave the TT of maize and enter the ovary cavity, almost the whole PT pathway is accomplished. The last remaining task for the tube is to reach the micropyle, enter the micropylar nucellus region and discharge the two sperm cells in the receptive synergid cell for double fertilization. It was found that, with the exception of micropylar short range guidance, PT guidance signals in the ovary cavity are exclusively controlled by the maternal sporophytic tissues of the ovule. From the above data, an up-to-date model of the PT pathway in maize was drawn analogous to the pathway in *A. thaliana* (Johnson and Preuss, 2002) and, based on the classification of Heslop-Harrison (1982), taking detailed anatomical surveys into account. As shown in Fig. 6, PT growth was divided in five phases: Phase I includes pollen grain capture, adhesion, hydration, and germination. This phase is mainly governed by general physiological parameters. In contrast to the SI-systems in the Brassicaceae and Solanaceae (Swanson *et al.*, 2004) species-specificity plays a minor role at this stage in maize and *T. dactyloides*, and does not represent a major hybridization barrier. Phase II has been further defined by the entry of the PT into the sporophytic tissue until it has reached the TT. This phase shows higher specificity and requires species-specific interactions between the male gametophytic and sporophytic cells. The TT cells probably generate species-preferential chemotropic guidance

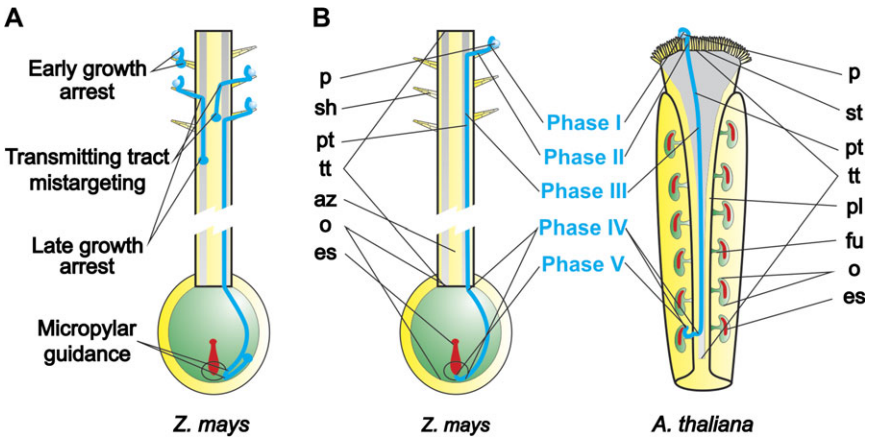

Fig. 6. Summary of comparative progamic pollen germination and growth in maize and *Arabidopsis*. Although anatomical structures are named differently, Phase I–III can be widely homologized between maize and *Arabidopsis*. (A) Scheme showing progamic pollen tube growth failures observed in this survey. Early growth arrest was observed with pollen of rice, *Poa*, *Lolium*, and *Arabidopsis*. Lily pollen did not germinate or stopped growth before leaving the silk hair. (B) Pollen tubes of these species arrested in Phase I (lily) or Phase II (most grasses and *Arabidopsis*). *Tripsacum* and incompatible maize pollen tubes show two linked phenomena. Many pollen tubes are mistargeted and grow outside the transmitting tract. Those pollen tubes which find their way into the transmitting tract stop growth after a few centimetres (Phase III). After passing the abscission zone in maize, pollen tubes reach the end of the transmitting tract and enter the ovarial cavity. Growth in the ovarial cavity of grasses and, accordingly, growth on the septum surface, funiculus, and ovule surface of *Arabidopsis* represent Phase IV. Whereas female gametophytes of *Arabidopsis* contribute to funicular and micropylar guidance, maize embryo sacs only provide micropylar guidance cues (Phase V). As a sporophytic default status, maize pollen tubes are guided towards the micropyle, but do not enter an area about 100 μm in diameter around the micropylar cone. Ovules are shown in green, transmitting tracts in grey, embryo sacs in red, and male gametophytes in blue. Abbreviations: az, abscission zone; es, embryo sac; fu, funiculus; o, ovule; p, pollen; pl, placenta; pt, pollen tube; sh, silk hair; st, stigma; tt, transmitting tract.

signals attracting the PT inside its intercellular spaces, preformed by the anatomy of silks and silk hairs. Phase III, stigmatic PT growth inside the TT, begins when PTs enter the TT and ends when they are leaving it towards the ovular cavity. Although this phase is believed to be regulated by tract geometry (Heslop-Harrison, 1982), it was found here that stigmatic PT growth depends on growth support and nutrients provided and was controlled by the sporophytic tissues, thus representing another hybridization barrier. During growth through the TT, PT–silk interactions cause degeneration of the abscission zone (AZ) proximal to the upper part of the ovary (style) once it was passed by the first 5–10 tubes (Heslop-Harrison *et al.*, 1985). This barrier to the entry of supernumerary tubes thus represents one of the components to avoid polyspermy. While the AZ degenerates, vascular bundles and, presumably TTs, are quickly interrupted. Injured TTs also lead to an inability of the PT to pass its destroyed tissue (Booy *et al.*, 1992). At the end of the TTs, PTs enter the ovular cavity in Phase IV. With the process of leaving the TT, anatomical and physiological aspects between *A. thaliana* and maize are different: in *A. thaliana*, PTs are directed from various positions of the TT towards the placental surface along the septum tissue. In grasses in general, and in maize in particular, TTs end blindly close to the upper ovary and PTs enter the ovary cavity by breaking through the inner epidermis of the ovary wall (Heslop-Harrison *et al.*, 1985). An additional difference between both model systems is that PTs of *A. thaliana* grow on the septum surface in an air-filled environment, whereas grass PTs remains constantly surrounded in the ovary cavity by the ovary and the inner integument cell walls. Here, the elongated cells of the inner integument are aligned towards the micropylar region providing an anatomical growth direction clue (Fig. 5F). Finally, in Phase V, *A. thaliana* PTs have to find and pass structures such as the funiculus, whereas grass PTs are immediately directed towards the micropylar cone after leaving the TT.

PTs of *A. thaliana* thus need more distinct signals including funicular and micropylar guidance in order to accomplish fertilization (Higashiyama and Hamamura, 2008). Both processes are thought to depend on signalling governed by the female gametophyte. By contrast, grass PTs only need to be guided to the micropyle towards the egg apparatus. The possibility that the egg apparatus itself and the synergids in particular, produce the respective chemotropic factors has been discussed for many years (van der Pluijm, 1964). Until now, only one short range guidance molecule has been discovered in maize (Márton *et al.*, 2005; Dresselhaus and Márton, 2009). ZmEA1 is secreted from the egg apparatus towards the cell walls of the micropylar nucellar cells and is able to attract maize pollen tubes *in vitro*. PTs in RNAi *ZmEA1*-knock down plants reach the micropylar cone up to a distance of about 100 μm, the same distance that has been described here for ovules lacking female gametophytes. In conclusion, progamic PT development is largely governed by interactions and communication between the male gametophyte and the surrounding sporophytic tissues. Pollen germination is relatively unspecific and seems to depend mainly on the environmental conditions at the silk surface. Initial growth of PTs between silk hair cells appears to be more specific and thus represent a first hybridization barrier. Pollen of more distantly related grass species arrest their growth in the silk hairs (second hybridization barrier), whereas PTs of maize and *T. dactyloides* are capable of growing further towards the TT. In incompatible cross-pollinations between maize and *T. dactyloides*, and in maize itself, PTs frequently fail to target the TT and growth is arrested after 4–6 cm pointing to a second and third hybridization barrier. This indicates a bias of species-specific recognition, nutrition, and guidance signalling between male gametophyte and sporophytic silk tissues. In maize, guidance by the female gametophyte is restricted to a small area around the micropyle and probably represents a fourth hybridization barrier analogous with findings in *Torenia fournieri* where, similar to polymorphic ZmEA1 protein (Márton *et al.*, 2005), polymorphic defensin-like proteins LURE1 and LURE2 control micropylar PT guidance in a species-specific manner (Okuda *et al.*, 2009). Our findings thus support the idea that pre-zygotic barriers to genetic exchange are stronger than post-zygotic barriers (Rieseberg and Willis, 2007; Widmer *et al.*, 2009) and thus also prepresent a major driving force for speciation in the grasses.

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