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REVIEW

Insights into the molecular control of cross-incompatibility in *Zea mays*

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Abstract

Gametophytic cross-incompatibility systems in corn have been the subject of genetic studies for more than a century. They have tremendous economic potential as a genetic mechanism for controlling fertilization without controlling pollination. Three major genetically distinct and functionally equivalent cross-incompatibility systems exist in *Zea mays*: *Ga1*, *Tcb1*, and *Ga2*. All three confer reproductive isolation between maize or teosinte varieties with different haplotypes at any one locus. These loci confer genetically separable functions to the silk and pollen: a female function that allows the silk to block fertilization by non-self-type pollen and a male function that overcomes the block of the female function from the same locus. Identification of some of these genes has shed light on the reproductive isolation they confer. The identification of both male and female factors as pectin methylesterases reveals the importance of pectin methylesterase activity in controlling the decision between pollen acceptance versus rejection, possibly by regulating the degree of methylesterification of the pollen tube cell wall. The appropriate level and spatial distribution of pectin methylesterification is critical for pollen tube growth and is affected by both pectin methylesterases and pectin methylesterase inhibitors. We present a molecular model that explains how cross-incompatibility systems may function that can be tested in *Zea* and uncharacterized cross-incompatibility systems. Molecular characterization of these loci in conjunction with further refinement of the underlying molecular and cellular mechanisms will allow researchers to bring new and powerful tools to bear on understanding reproductive isolation in *Zea mays* and related species.

Keywords Cross-incompatibility · Pectin methylesterase · *Zea mays* · Pollen · Pistil · Reproductive isolation

Overview of gametophytic cross-incompatibility

Many plant species have adopted unilateral cross-incompatibility (CI) systems to promote self-propagation (Lewis and Crowe 1958). Most maize (*Zea mays* L.) plants can freely cross-pollinate each other, facilitating propagation of the species in highly heterozygous form and allowing plants to benefit from heterosis. However, gametophytic CI (also known as dent sterility in popcorn) systems in maize interfere with this free cross-pollination by preventing fertilization of plants carrying a CI system by pollen of plants that do not, while allowing reciprocal crosses to be successful (Baltazar et al. 2005; Kermicle and Evans 2010; Schwartz 1950). The fertilization barrier provided by these systems may have contributed to the evolution of maize and the preservation of some strains of teosinte by providing reproductive isolation (Kermicle 2006). Gametophytic CI systems have been used to provide reproductive isolation in commercial maize production. Understanding their function may provide fundamental insights into pollen-pistil interactions and lead to applications that impact seed production and genetic purity of maize products.

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Economic importance of maize pollination

Maize is a staple crop for billions of people and is produced on every continent except Antarctica. It is used for food, feed and fuel. Some cultures were so tightly linked to maize production that it took on a religious significance (Taube 1996; Miller and Taube 1993; Taube 1985). Unlike the other major cereals wheat and rice, maize is naturally a cross-pollinated species. It is produced as open-pollinated varieties in many parts of the world and as hybrids of pure lines in others. Many specialty varieties of maize have been developed, including colored varieties, varieties with modified starch such as sweet and waxy corn, popcorn, and silage corn (Scott et al. 2019). A better understanding of the process of fertilization in maize is important for improvement of maize and global food security. Understanding the molecular mechanism of gametophytic incompatibility systems is an important contribution to understanding the process of fertilization and may facilitate improvements in seed production, separation of varieties with unique traits, and access to divergent germplasm.

Maize pollination

In maize, male and female flower organs are segregated in the tassel and ear, respectively, allowing for high rates of cross-pollination. The silk, the stigma and style of the maize pistil, develops and elongates from each ovary on the ear. Pollination occurs when (typically for grasses) wind-carried pollen grains land on the silks. Fertilization requires transport of the sperm cells by the pollen tube from point of pollination to the ovary where fertilization of an ovule occurs. This process requires a complex interaction between the pollen tube of the male gametophyte and sporophytic and gametophytic tissue in the female. This process is divided into five stages [reviewed by (Dresselhaus and Franklin-Tong 2013)]. In Phase I, pollen grains adhere to the surface of the stigma, hydrate and germinate on the stigmatic tissue surface to produce a specialized cell called a pollen tube that ultimately carries the sperm cells to the embryo sac. In Phase II, pollen tubes elongate, invading the tissue of the silk in the intercellular space toward the transmitting tract, a specialized tissue that connects the stigma, style and ovary. The pollen tubes themselves do not have the resources required to elongate the entire length of the silk, and receive growth support from the nutrient-rich extracellular matrix of the transmitting tract. In phase III, pollen tubes grow along the transmitting tract toward the ovary at the base of the carpel. In phase IV, pollen tubes leave the transmitting tract and are directed toward the ovule micropylar region. In phase V, pollen tube growth is directed toward the egg apparatus. Upon reaching the egg apparatus the pollen tube ruptures, releasing the two sperm cells that fertilize the female gametes, the egg and central cell, to initiate seed development. In maize, typically, multiple pollen grains land on a single silk and produce pollen tubes, and only the first pollen tube to reach and fertilize the embryo sac will produce an offspring. Slower growing pollen tubes are eliminated by competition.

Pollen tubes elongate by tip growth (Steer and Steer 1989), which allows the pollen vegetative cell to travel 30 cm or more to deliver sperm cells to the embryo sac for fertilization. Key components of the cellular machinery regulating this polar cell growth include ion dynamics (Rathore et al. 1991; Holdaway-Clarke and Hepler 2003), actin filaments and regulatory proteins (Vidali and Hepler 2001), polar vesicle trafficking and the Rac/Rop small GTPase (de Graaf et al. 2005). This tip-localized growth also allows the fast-growing pollen tube cell to communicate and respond to the surrounding female cells effectively. Pollen tube growth is also regulated by extra cellular matrix components, including various signaling mechanisms of the stylar region. To support polarized cell growth, the tube cell must be strong enough laterally to withstand the internal turgor pressure and plastic enough in the apical region to allow for the incorporation of new membrane and cell wall material for tip growth [reviewed by (Zonia 2010; Dresselhaus and Franklin-Tong 2013)].

The apical region of the pollen tube wall is composed of a single layer of pectin (Geitmann et al. 1995; Taylor and Hepler 1997) and is devoid of callose, cellulose and hemicellulose (Ferguson et al. 1998). Pectins are synthesized and methyl esterified in Golgi and secreted into the cell wall in a highly methylesterified state (Micheli 2001; Sterling et al. 2001). Homogalacturonans (HGA) are a major component of tube cell wall pectins and greatly impact the biophysical properties of the cell wall. Pectin methylesterases (PMEs) de-esterify adjacent HGA by removing methyl groups and leaving free carboxyl groups on HGA residues. These free carboxyl groups on adjacent HGA chains are cross-linked by calcium causing pectin gelation and thereby cell wall rigidification (Willats et al. 2001). Pectins are differentially esterified in the apical and lateral regions of the tube cell wall after their secretion. The apical region of pollen tube tip is enriched for esterified pectins, providing plasticity required for tip growth while the lateral cell wall region is enriched for de-esterified pectins, providing rigidity required for withstanding pressure (Bosch et al. 2005). Therefore, spatial and temporal regulation of the degree of methyl esterification at the tip of the pollen tube and along the shank of the pollen tube are necessary for polarized tip growth and are achieved by localized regulation of PME enzyme activity

that catalyzes de-methylesterification of HGA (Röckel et al. 2008; Tian et al. 2006). Previously, several studies have demonstrated that the activity of PMEs is regulated locally by a proteinaceous inhibitor called pectin methylesterase inhibitor (PMEI) [reviewed by (Jolie et al. 2010)], and the specific PME-PMEI interaction leads to proper pollen tube growth. A balance between PME and PMEI activities play a central role in cell wall rigidification.

Gametophytic cross-incompatibility systems of maize

Several gametophytic factors in maize are known to interfere with cross-pollination. The most well-characterized of these factors are *Gametophyte factor1* (*Ga1*), *Gametophyte factor2* (*Ga2*) and *Teosinte crossing barrier1* (*Tcb1*) (Fig. 1). These cross-incompatibility systems are functionally equivalent but genetically distinct. Each system is characterized by two functions: (1) a female activity expressed in the pistil (silk) that acts as a barrier to fertilization by pollen that does not carry a specific allele from that system; and (2) a male activity expressed in pollen that allows that pollen grain to overcome the pollen exclusion barrier of its own system. The male and female functions are tightly linked genetically and typically inherit as a single unit. In incompatible pollinations mediated by all three cross-incompatibility systems, pollen germinates and the pollen tube penetrates the silk but pollen tube growth is slowed and eventually ceases in phase III (Lu et al. 2014; House and Nelson 1958; Lausser et al. 2010). Another interesting, and likely related feature, is that when these barriers are attenuated and incomplete, partial seed set occurs in the tip region of the ear which has the shortest silks (Fig. 1d).

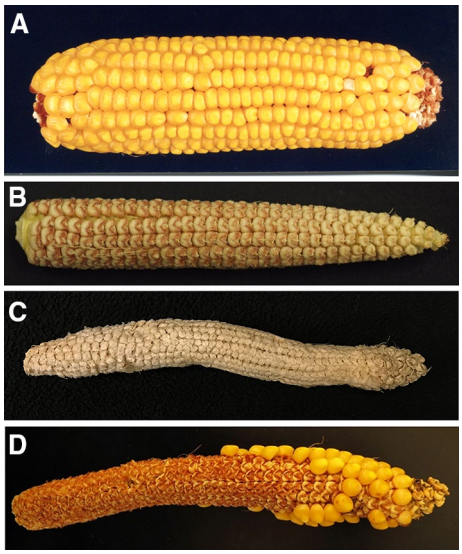

Fig. 1 Ears from compatible (**a**) and incompatible (**b**–**d**) pollinations. The tip of the ear is on the right. All ears were crossed with pollen of the same genotype, *ga1 ga2 tcb1*, lacking any CI functions. **a** *ga1 ga2 tcb1* female x *ga1 ga2 tcb1* male. **b** *ga1 ga2 Tcb1-s* female x *ga1 ga2 tcb1* male. **c** *Ga1-s ga2 tcb1* female x *ga1 ga2 tcb1* male. **d** *ga1 Ga2-s tcb1* female x *ga1 ga2 tcb1* male. In crosses with partial seed set rather than zero seed set, seed is set near the tip of the ear with all three systems (as shown here for *Ga2-s* in **d**)

While scientists have studied gametophytic incompatibility systems for more than 100 years (Correns 1901), the causative genes and the underlying molecular and cellular mechanisms are just now being elucidated. Study of gametophytic incompatibility systems has been hindered by two factors. First, it is difficult to observe and measure the pollen exclusion phenotype. Second, genomic studies of varieties carrying gametophytic incompatibility systems have lagged behind those of other maize varieties. This problem is exacerbated by the diversity of gene content in different maize varieties (Fu and Dooner 2002). Genetic evidence demonstrates that cross-incompatibility systems are absent from most strains of temperate-adapted dent corn. The publication of a complete genome sequence of the *Ga1-s*/*Ga-s* line HP301 (https://nam-genomes.org/) provides a valuable resource to understand the genomic organization of this complicated locus.

Genetics of gametophytic cross-incompatibility in *Zea mays*

Dent sterility is a gametophytic cross-incompatibility system exhibited by many popcorn strains that prevents cross-fertilization by pollen of most dent varieties. Dent sterility is genetically regulated by a locus known as *gametophyte factor1* (*ga1*) (Mangelsdorf and Jones 1926) and is the best characterized of the gametophytic incompatibility systems. There are at least three different haplotypes at the Ga1 locus: *Ga1-s*, *Ga1-m* and *ga1*. Haplotypes are functionally classified based on the presence or absence of the male or female functions. Many popcorn varieties have the *Ga1-s* haplotype, which carries both male and female functions and therefore cannot be pollinated by dent pollen, but can pollinate other *Ga1-s* plants. Most dent varieties carry the *ga1* haplotype that lacks male and female *Ga1-s* functions and so accept all types of pollen but cannot pollinate silks carrying *Ga1-s*. Plants with the *Ga1-m* haplotype have the male function but lack the female function so these plants can fertilize and be fertilized by *Ga1-s*, *Ga1-m* and *ga1*. Parallel haplotypes are also found at the *tcb1* and *ga2* loci: *Tcb1-s* and *Ga2-s* with male and female functions, *Tcb1-m* and *Ga2-m* with male functions only, and *tcb1* and *ga2* with neither male nor female functions. The genotype of most temperate maize lines is *ga1 ga2 tcb1* with no cross-incompatibility functions. For all three loci, the male function is conditioned

gametophytically so that function of pollen grains from heterozygotes depends on which haplotype is present in the haploid pollen while the female function is expressed in silks, a sporophytic diploid tissue. This can lead to segregation distortion of the locus itself as well as linked loci in the progeny of heterozygous males.

The identification of *Ga1-m* (Jimenez and Nelson 1965) provided a critical insight into the utility of *Ga1-s* as a pollen exclusion barrier. It is important to understand how widespread this allele is because of its ability to overcome the reproductive barrier imposed by *Ga1-s*. A survey of diverse sets of germplasm shows that the *Ga1-m* haplotype is present in many lines, particularly in tropical germplasm (Kermicle 2006; Jones and Goodman 2018; Kermicle and Evans 2010; Kermicle et al. 2006). Similarly, *Ga2-m* is found in a variety of tropical maize germplasm as well, and *Ga1-m*, *Ga2-m*, and *Tcb1-m* are found in populations of both *parviglumis* and *mexicana* teosinte (Kermicle et al. 2006; Kermicle 2006; Jones and Goodman 2018). Because many breeding programs incorporate diverse germplasm from many sources, and some breeding programs depend on cross-incompatibility to maintain their identity (e.g., popcorn breeding), the existence of m-type haplotypes has the potential to reduce the ability to exclude unwanted germplasm using a gametophytic incompatibility system. This concern led to identification of additional lines carrying an m-type haplotype for the *Ga1*, *Ga2*, and *Tcb1* systems (Jones and Goodman 2018) and identification of a Maiz Dulce line carrying *Tcb1-s* (Jones et al. 2015, 2016; Lu et al. 2019).

The identification of *m-type* alleles is also critical for understanding the molecular function of gametophytic incompatibility systems because it demonstrates that the male and female functions of *Ga1-s* are genetically separable, even though they map to the same genetic locus. Fine mapping of the *Tcb1-s* locus demonstrated that the male and female factors could be separated by recombination to obtain either *Tcb1-male* or *Tcb1-female* haplotypes (Lu et al. 2014). This supports the model that the male and female functions are encoded by separate genes for each locus. So, there are four possible haplotypes for each of these barrier loci. For example, in the *Tcb1* locus, the four haplotypes are: (1) *Tcb1-m Tcb1-f* (equals *Tcb1-s*, has both male and female functions); (2) *Tcb1-m tcb1-f* (has only the male function, able to pollinate *Tcb1-s* silks, but also receptive to *tcb1* pollen); (3) *tcb1-m Tcb1-f* (has only the female function, pollen is blocked by *Tcb1-s* silks, but silks block *tcb1* pollen) and (4) *tcb1-m tcb1-f* (has neither male or female function, standard maize type, referred to as the *tcb1* haplotype) (Lu et al. 2014). Among these four haplotypes, *Tcb1-m Tcb1-f*, *Tcb1-m tcb1-f* and *tcb1-m tcb1-f* types were found in natural teosinte populations, and the *tcb1-m tcb1-f* haplotype is found in standard north American maize lines (Lu et al. 2014). The *tcb1-m Tcb1-f* haplotype has been identified by recombination within the *Tcb1-s* haplotype, has not been found in the wild, and is self-incompatible since its pollen is similar to that of the *tcb1* haplotype and cannot function on the *Tcb1-f* silks.

An important point of emphasis is that the three CI systems are mostly incompatible with each other (Fig. 2). For example, *Tcb1-s* females can exclude *Ga1-s* pollen in favor of *Tcb1-s* pollen, and when the barrier is at full strength no seed is set in *Tcb1-s* females pollinated by *Ga1-s*. This is true in all combinations of two CI factors crossed in either direction. Consequently, these three loci are not simply the same pair of genes at different chromosomal locations in different lines. However, in cases where the barrier is not full-strength (whether they are attenuated by *cis* or *trans* effects is not always known), partial seed set is possible, and there is a slight advantage to having a mismatched CI pollen function over no CI pollen function (Kermicle and Evans 2010; Evans and Kermicle 2001). For example, on *Tcb1-s ga1 ga2* or *tcb1 ga1 Ga2-s* females, *tcb1 Ga1-s ga2* pollen will produce proportionally more seeds than *tcb1 ga1 ga2* pollen when the two are in competition (i.e., to achieve fertilization it is better to have a poorly matched CI male function than none at all). This is consistent with a model in which all three loci encode functionally related but non-identical proteins and the male and female factors can distinguish their cognate partners from those of the other two loci.

The genotype-specific rejection of pollen by the pistil in these CI systems is reminiscent of Self-Incompatible (SI) systems. SI can be found in many plant taxa including

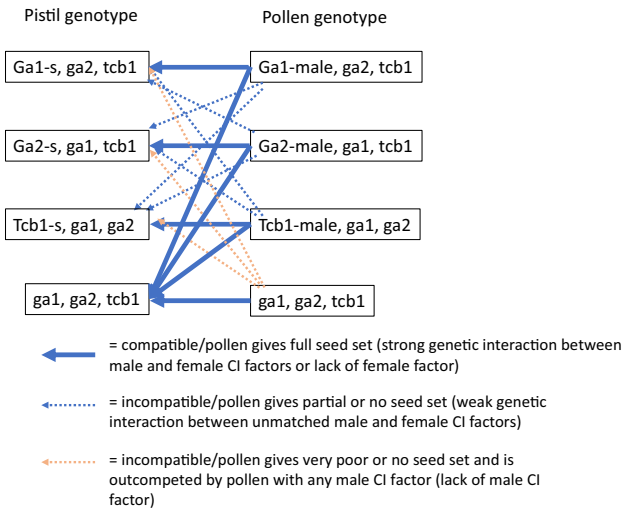

Fig. 2 Diagram of success versus failure of pollination between different CI type females and males. A cognate pair match of male and female factors or the absence of female factors gives full seed set. The lack of any male factor when a female factor is present in a cross gives a failed pollination. A mismatch between the male and female factors, while typically poor, is better than having no male CI factor at all

Brassicaceae, Solanaceae, Papavaraceae, and Poaceae (Jany et al. 2019; Langridge and Baumann 2008; Takayama and Isogai 2005). SI systems have many features in common with the gametophytic CI systems of maize. They function through the interaction of two components, one present in pistils and the other present in pollen that interact to prevent fertilization. Like the maize systems characterized to date, the two components are typically tightly linked genetically to form a complex multifunctional locus [although some multi-locus systems are found in the Poaceae (Langridge and Baumann 2008)]. Intriguingly, interspecific CI systems within these taxa are often unilateral, similar to maize CI systems, and can rely on some of the same genes as used in SI (Hancock et al. 2003; Kitashiba and Nasrallah 2014), allowing development of a model for a universal pathway that controls both self- and interspecific incompatibility within a taxon.

Three well-studied mechanisms, the gametophytic SI systems in Solanaceae and Papaveraceae and the sporophytic SI system in Brassicaceae, use different molecular mechanisms to prevent post-pollination fertilization (McClure and Franklin-Tong 2006; Takayama and Isogai 2005; Kumar and McClure 2010; Wang et al. 2019). In sporophytic SI in Brassicaceae direct interaction between the female and male SI proteins, S-LOCUS RECEPTOR KINASE (SRK) and S-LOCUS PROTEIN11/S-LOCUS CYSTEINE-RICH (SP11/SCR), respectively, initiates a signal transduction cascade leading to the arrest of the self-pollen (Takayama and Isogai 2005). In gametophytic SI in Papaveraceae, the female factor, a small, secreted peptide named PrsS (Papaver rhoeas stigma S determinant) interacts with the male factor, plasma membrane protein named PrpS (Papaver rhoeas pollen S), leading to programmed cell death (Wang et al. 2019). In the Solanaceae, the female S-RNase (McClure et al. 1989) is toxic to self-pollen after its uptake by the pollen tube in incompatible crosses, while in compatible crosses, the S-RNase is degraded inside the pollen tube in a pathway dependent on the non-self allele of the male component, the S-LOCUS F-box gene (Sijacic et al. 2004; Luu et al. 2000; Gray et al. 1991). Common features of these SI systems is that there are many alleles and that the male and female S-LOCUS proteins interact directly. This interaction between male and female proteins is haplotype-specific in Brassica and Papaver leading to pollen failure, while S-RNase interacts with multiple alleles of the S-LOCUS F-box but is not degraded by the allele of the same haplotype. In all cases, haplotype-specificity is determined by direct interaction between the male and female components. One feature that distinguishes the SI systems from the maize CI systems is the difference in allelic diversity; there are many functionally distinct alleles of SI loci but the maize CI systems lack that diversity.

Molecular studies of gametophytic cross-incompatibility systems of maize

Combinations of genetics, genomics, transcriptomics, and functional tests have identified a few of these genes (Table 1). An RNA-Seq experiment identified a PME gene, *ZmPME3*, with abundant expression in *Ga1-s* silks and much lower expression in *ga1* genotypes as a candidate gene for the female function of the *Ga1-s* locus (Moran Lauter et al. 2017). This gene maps within a cluster of *PME* pseudo genes at the *Ga1* locus, but is only found in an active form in *Ga1-s* genotypes. The *ZmPME3* RNA and protein are found in *Ga1-s* silks, and interestingly this gene has a frame-shift mutation in a subset of *Ga1-m* haplotypes, while other lines with a *Ga1-m* phenotype (e.g., NC350, NC358, CML333, CML52, Tzi8 (Jones and Goodman 2018)) have an intact *ZmPME3* gene (unpublished observations). While on its own this may suggest *ZmPME3* does not encode *Ga1-female*, mutagenesis, mapping, and RNA-Seq identified a similar PME-encoding gene as the *Tcb1-f* gene, and some *Tcb1-m* lines arise from *Tcb1-s* by apparent silencing of the *Tcb1-f* gene (Lu et al. 2019). A second potential explanation for these results is that modifier genes interact with *ZmPME3* rendering it non-functional in these lines. Both of *Tcb1-f* and *Ga1-f/ZmPME3* are most similar to PME38 in Arabidopsis. Both are predicted to be secreted and neither contains a pro-domain with PME inhibitor (PMEI) similarity (nor do other PME proteins in the PME38 branch of the family) (Fig. 3). The TCB1-F and GA1-F/ZMPME3 proteins only have nine amino acid differences from each other. The limited cross-compatibility between the *Ga1* and *Tcb1* systems suggests that these nine differences are sufficient to functionally differentiate the *Ga1* and *Tcb1* systems. Based on nucleotide substitution rate and calculated splitting time between *mexicana* and *parviglumis*, it was estimated that *Tcb1-f* and *Ga1-f/ZmPme3* diverged before the split between *mexicana* and *parviglumis* (Lu et al. 2019). Both *mexicana* and *parviglumis* are polymorphic at all CI loci with *m-type*, *s-type*, and non-functional, standard maize-like haplotypes of *Ga1*, *Ga2*, and *Tcb1* found in wild populations (Kermicle 2006; Kermicle and Evans 2010; Kermicle et al. 2006).

Using map-based cloning, the male component of *Ga1-s* was identified as a pollen-specific pectin methylesterase

Table 1 Cloned cross-incompatilibity genes of *Zea mays*

CI Gene	Other names	Protein encoded	References
Ga1-female	*ZmPME3*	PME38-LIKE	Moran Lauter et al. (2017)
Ga1-male	*Ga1P*	QRT1-LIKE	Zhang et al. (2018)
Tcb1-female		PME38-LIKE	Lu et al. (2019)

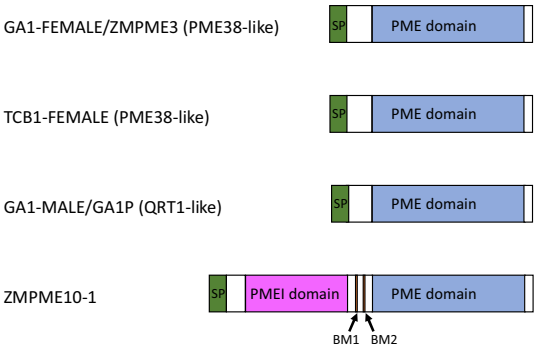

Fig. 3 Protein structure of cloned CI PME genes and the interacting ZMPME10-1 protein. The mature PME domain is shown in blue. Signal peptides (SP) are shown in green. The PMEI region of the pro-domain is shown in pink. Binding motifs 1 and 2 (BM1 and BM2), target sites for cleavage, are indicated in ZMPME10-1

called *Ga1P* (Zhang et al. 2018). *Ga1P* is in a different PME clade than *Ga1-f* or *Tcb1-f*, is most similar to Arabidopsis *QUARTET1* (*QRT1*) and, like *Ga1-f* and *Tcb1-f*, lacks a PMEI-like pro-domain (Fig. 3). Transgenic plants expressing *Ga1P* were able to overcome the pollination barrier imposed by *Ga1-s*, establishing *Ga1P* as the male factor of the *Ga1* locus. A simple model for the CI locus specificity for male factors to overcome female barriers (i.e., *Ga1-m* pollen fertilizes *Ga1-s* females efficiently but not *Tcb1-s* or *Ga2-s* females) is that the male and female proteins interact directly, changing PME activity levels, and that both male and female factors are sufficiently diverged between CI systems that cross-CI system interactions are poor. *Ga1P* was shown to interact with another type of PME/PMEI called ZmPME10-1 that contains a N-terminal pro-domain (Fig. 3) and is expressed in *Ga1-s* and *ga1* pollen. The authors suggest that these two enzymes "may form a protein complex to maintain the equilibrium of the apical cell wall dynamics during pollen tube growth" (Zhang et al. 2018).

PMEs/PMEIs in pollen tube growth

Pectin is a major component of plant cell walls. As an enzyme to modify the chemical properties of pectin on the cell walls, plant PMEs play many physiological roles (Micheli 2001), including in embryo development (Levesque-Tremblay et al. 2015), phyllotaxis (Peaucelle et al. 2008), anisotropic hypocotyl growth during germination (Bou Daher et al. 2018; Derbyshire et al. 2007), plant immune responses (Bethke et al. 2014), and pollen tube growth in successful reproduction (Bosch and Hepler 2006; Jiang et al. 2005; Bosch et al. 2005). Besides being regulated by different physiological environments, including pH and cations (Goldberg et al. 2001; Denes et al. 2000; Moustacas et al. 1991), PME activity can also be regulated molecularly by PMEI proteins (Jolie et al. 2010; Balestrieri et al. 1990). PMEs can be broadly categorized into two groups, with group 1 containing only a PME domain while group 2 has an N-terminal pro-domain containing a PMEI domain (Pelloux et al. 2007). Typically, for group 2 PMEs, the PMEI domain is proteolytically cleaved from the PME domain prior to secretion, and secretion of the PME depends upon this cleavage (Wolf et al. 2009). Cleavage depends on two four-residue motifs, binding motif 1 (BM1) and BM2 between the PMEI domain and the PME domain. This would likely lead to secretion of separate PME and PMEI proteins simultaneously. The interaction mentioned above between ZMPME10-1 and GA1-MALE/GA1P is with the PME domain of ZMPME10-1. This fact, coupled with the fact that the BM1 and BM2 sites are intact in ZMPME10-1 (Fig. 3) suggests that extracellular complexes with GA1-M/GA1P would not include the PMEI domain of ZMPME10-1. Interestingly, the origin of PMEs is associated with accelerated pollen tube growth in angiosperms (Wallace and Williams 2017), which in turn has been proposed as one of the evolutionary novelties that make angiosperms so successful and dominant (Williams 2008; Mulcahy 1979). PMEs could be key players in plant reproductive isolation by modifying the pollen tube cell wall (Lu et al. 2019), adding fresh perspectives on the roles PMEs play in plant evolutionary biology.

The Arabidopsis genome has 66 PME genes (Pelloux et al. 2007), at least 18 of which are expressed in pollen (Pina et al. 2005). Two Arabidopsis PMEI genes, *AtPMEI1* and *AtPMEI2*, selectively express in pollen and inhibit PME activity from a wide range of species (Wolf et al. 2003; Raiola et al. 2004; Lionetti et al. 2007). *AtPMEI1*, *AtPMEI2* and *AtPPME1* genes showed distinct distribution patterns when they were transiently expressed in Nicotiana pollen tubes. *AtPMEI1* and *AtPMEI2* are localized to the tube apical cell wall while *AtPPME1* was found both in the tip apex and in the pollen tube shank. In addition to their specific distribution patterns, AtPMEI and AtPPME1 genes differentially regulated pollen tube growth. AtPMEI genes promoted pollen tube growth whereas AtPPME1 restricted tube growth. Based on the distinct localization pattern and differential regulation of tube growth, it has been proposed that *AtPMEI2* promotes tube growth by locally inhibiting PME activity at the tube tip (Röckel et al. 2008). Another Arabidopsis PME gene, *VANGUARD1* (*VGD1*), which is specifically expressed in the pollen grain and pollen tube, has been shown to be necessary for pollen tube growth in the style and transmitting tract. It was hypothesized that the loss of *VGD1* function may cause reduced de-methylesterification of pectin in the tube cell wall and reduce pollen tube growth by reducing pollen tube wall strength or reducing interactions between the pollen tube and the transmitting tract extracellular

matrix (Jiang et al. 2005). The Arabidopsis *QRT1* pectin methylesterase, in conjunction with the polygalacturonases, *QRT2* and *QRT3*, promotes pollen mother cell wall breakdown and pollen tetrad separation (Rhee et al. 2003; Rhee and Somerville 1998; Francis et al. 2006; Ogawa et al. 2009; Preuss et al. 1994).

BoPMEI1, a *Brassica oleracea* PMEI gene, was found to be expressed specifically in pollen grains and pollen tubes of cabbage maintainer lines but not in male sterile lines. The antisense expression of *BoPMEI1* in Arabidopsis reduced the expression of its Arabidopsis ortholog and resulted in reduced pollen tube growth and poor seed set (Zhang et al. 2010). Several species-specific PME genes, *Bp19* from Brassica napus, *PPE1* from *Petunia inflata*, *ZmC5* from Maize and *OsPME1* from Rice, specifically expressing in the later stages of pollen development have been identified (Mu et al. 1994; Wakeley et al. 1998; Kanneganti and Gupta 2009; Alabani et al. 1991). However, these genes have not been functionally characterized.

In maize inbred B73, there are 43 PMEs (20 of which encode group 2 PMEs with a PMEI containing prodomain) and an additional 49 PMEIs (Zhang et al. 2019). At least nine of the PMEs are expressed in pollen. Maize *ZmPMEI1* has specific expression in both male (pollen grain) and female (embryo sac) gametophytes and acts as an inhibitor of PME (Woriedh et al. 2013). *ZmPMEI1* is expressed in the pollen grain and pollen tube during its entry into silks and growth in the transmitting tract. In the pollen tube, *ZmPMEI1*-EGFP fusion proteins are abundant in the vegetative cell and growing pollen tubes. External application of *ZmPMEI1* protein did not affect tube tip growth but caused swelling and tube burst in the subapical region of the pollen tube.

Taken together, an abundance of data from maize and other species suggests that the proper balance of methylesterification/de-methylesterification is critical for pollen tube function. Too much de-methylesterification by PMEs causes excessive pollen tube stiffening, blocking pollen tube elongation and leading to pollen failure (Bosch et al. 2005). Too little de-methylesterification (particularly along the shank of the growing pollen tube) can lead to pollen tube rupture and thus also to failure (Woriedh et al. 2013). Consequently, increased activity of either PME or PMEI in the appropriate location would likely be sufficient to prevent fertilization by the targeted pollen tube. An optimal level of PME activity in the extracellular space containing the growing pollen tube wall is required for proper pollen tube growth. Modulation of PME activity through expression of various combinations PMEs and PMEIs may be a mechanism for controlling pollen tube growth. A growing body of evidence suggests that this mechanism may be a common feature of gametophytic incompatibility systems in *Zea mays*.

Molecular model to explain how PMEs may function in gametophytic cross-incompatibility

Recent studies clearly establish a role for PME activity in CI in *Zea mays* (Zhang et al. 2018; Moran Lauter et al. 2017; Lu et al. 2019). Based on this and additional genetic evidence, we propose a model for how PME activity may be involved in CI systems (Fig. 4). The central feature of the model is that compatible pollinations require an optimal level of PME activity, and this level is modulated by specific interactions between silk-expressed genes and pollen tube-expressed genes. This level is mediated by PMEs and/or PMEIs, some of which, like ZmPME10-1, are expressed in the pollen regardless of CI genotype (Zhang et al. 2018). Blocking pollen tube elongation by stiffening the cell walls of the pistil itself through its own CI PME (in *Ga1-s* or *Tcb1-s*) is considered less likely because the *Ga1-f* and *Tcb1-f* PME factors are expressed in the silks prior to pollination, and so the GA1-M protein (or a complex of GA1-M with ZmPME10-1 or other proteins) from the compatible *Ga1-m* pollen is unlikely to inhibit or overcome modifications of the silk pectin cell walls that occurred before its arrival. Additionally, modifying the pistil pectin wall by the male factor is unlikely to allow for the male factor of one locus to be specific for its female partner (i.e., modifying the cell walls of its partner CI female without affecting other CI females).

In incompatible crosses (*Tcb1-S* x *tcb1* or *Ga1-s* x *ga1*), the presence of the female factor in the silk could cause pollen tube growth arrest by altering the spatial distribution of PME activity and changing the methylesterification state of pectin in growing pollen tubes (Fig. 2B). Too much PME activity at the pollen tube tip can prevent tube elongation (Bosch et al. 2005), and too little PME activity below the tip of the pollen tube can lead to tube rupture (Woriedh et al. 2013). In compatible crosses between plants with functional CI systems, the pollen factor (GA1-M/Ga1P in the Ga1 system) may overcome the barrier imposed by the silk factor by a forming a complex with its cognate female factor preventing the female factor from disrupting PME activity levels. This interaction would be required for restoration of optimal PME activity level and would be less favorable between CI systems. This allows pollen containing an active CI system to pollinate plants lacking such a system because the male factor does not alter PME activity levels in the absence of the female factor. The model suggests that the pollen and silk factors have specific antagonistic functions, with the male protein inhibiting the function of the female, although PME activity has not been confirmed for any of the pollen or silk factors characterized to date. The function of the male

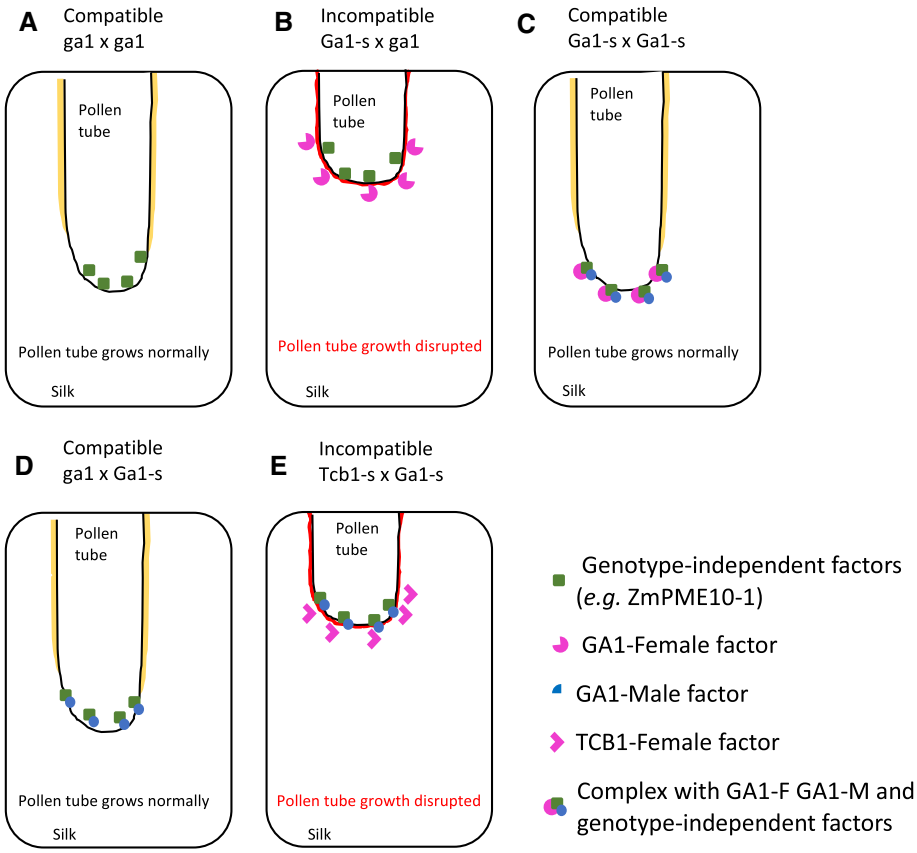

Fig. 4 Molecular model for PME-based unilateral cross-incompatibility. The growing point of the pollen tube in the silk is shown. Presence or absence of yellow shading of the pollen tube wall indicates variation in the degree of methylesterification of the pollen tube wall that is required for proper pollen tube growth (with yellow representing more de-esterification). Red shading of the pollen tube cell wall indicates a degree of methylesterification not suitable for proper pollen tube growth. **a**. When no CI systems are present endogenous enzymes such as ZmPME10-1 (Zhang et al., 2018) provide correct levels of PME activity to allow normal pollen tube growth. **b**. In incompatible reactions, the female factor (e.g., ZmPME3/Ga1-F in *Ga1-S*) alters PME activity so the pollen tube no longer grows normally. **c**. In compatible reactions involving a CI system, the action of the female factor is countered by the action of the male factor (Ga1P/GA1-M in the *Ga1-s* system, Zhang et al., 2018) that is carried by pollen. A CI locus-specific complex including male and female factors prevents PME activity disruption and allows normal pollen tube growth. This interaction may involve endogenous enzymes as well (Zhang et al., 2018). **d**. Pollen from plants containing a CI system can pollinate plants without a CI system because the pollen factor action is dependent on a specific interaction with the female factor. In the absence of the female factor, the pollen factor has no effect. **e**. Crosses between two different incompatibility systems fail because a complex including the male and female factors of different systems forms poorly. Yellow = pollen tube shank with high degree of de-methylesterified pectin compared to the pollen tube tip. Red = pollen tube wall with abnormal level or distribution of de-methylesterified pectin

factor could be to inactivate the female factor by forming a non-functional complex or by preventing the female factor from interfering with other proteins. Interestingly, the interaction of GA1-M/GA1P with genotype-independent proteins (Zhang et al., 2018) raises the intriguing possibility that other proteins may be recruited to participate in restoring optimum PME activity levels. This model also explains the outcome of crosses involving m-type alleles. The pollen of these plants possesses the male factor so it can overcome the barrier imposed by the female factor as described above. Because they lack a pistil-expressed CI PME, m-type plants can be pollinated by genotypes lacking a CI system. Finally, they can pollinate plants lacking a female factor because the male factor by itself does not alter PME activity levels. A model including inhibition of the female factor by association with the male factor is analogous to that of SI systems, in which specificity is determined by a combination of interaction and the polymorphisms between haplotypes (between loci here in *Zea* and between alleles in the SI systems).

Methylesterified pectins (as revealed by LM20 antibody labeling) were higher on or around the rejected *ga1* pollen tubes growing in *Ga1-s* silks than the compatible *Ga1-s* pollen tubes (Zhang et al. 2018). In contrast to this result, methylesterified pectin is highest (and de-methylesterified pectin lowest) at the pollen tube tip during normal pollen

tube growth in vitro (Bosch et al. 2005). Higher-resolution analysis of this distribution will help determine if this is affecting the subapical domain of the pollen tube (which could lead to tube rupture) or the whole pollen tube or the surrounding cells of the silk. Also, determination of the ratio of methylesterified and de-methylesterified pectins in different regions of the pollen tube during pollen rejection in incompatible crosses is essential to elucidate the molecular mechanism of pollen rejection. These studies would help to determine whether pollen rejection is associated with weakening or solidifying different regions of the pollen tube pectin wall.

Future directions

With the cloning of both the male and female genes in these crossing barrier systems in *Zea mays*, new exciting hypotheses can be tested in two areas of research: (1) the cellular and molecular mechanisms of these barriers; and (2) the prevalence of these mechanisms and their associations with speciation/reproductive isolation events in the grass family. Study of these barrier systems in *Zea mays* not only enhances our understanding of basic biological principles like cell signaling and polar cell growth, but will also shed light on the central problem of speciation, the origin and mechanism of reproductive isolation (Dobzhansky 1937).

Accordingly, new tools will need to be developed. For studies of cell and molecular biology of the PME-based CI, an in vitro system in which pollen tube growth inhibition can be recapitulated in the presence of the protein product of the female barrier gene will be a useful platform. This system will allow detailed qualitative and quantitative analysis of the dynamics and interactions of the male and female proteins and the modification of pollen tube cell walls (including their physical properties and methylesterification state) using protein labeling, cell wall staining, live-cell imaging, and atomic force microscopy. One advantage of this CI system for these studies is that the effects of the female component on pollen tube rejection/growth inhibition is genotype specific. This system would allow comparison of the effects of the ZMPME3 protein on compatible pollen (*Ga1-s*) and incompatible pollen (*ga1* or *Tcb1-m* or *Ga2-m*). Other genes required for the function of the barrier can also be identified from forward or reverse genetic screens (such as testing whether or not *ZmPME10-1* is required for either the barrier or for the ability of *Ga1-m* pollen to overcome the *Ga1-s* barrier).

To study the evolutionary significance of this PME-mediated barrier system, genomes of properly selected branches in the grass family, or other angiosperms, can be queried for the presences of similar CI systems. The first step would be to search for the existence of pairs of homologs of the *Zea mays* barrier genes. Combined with gene modification (gene knockout or transformation), actual barrier activities can then be tested in more species. The current prediction is that *Tcb1-s* and *Ga2-s*, like *Ga1-s*, consist of a *female/PME38-like* and *male/QRT1-like* gene pair because of the genetic evidence for partial interaction across CI loci, and also that these three loci are divergent enough to interact more poorly across barrier systems than with their cognate partner. Interestingly, there is a *PME38-like QRT1-like* gene pair near the location of the *Ga2* locus in the B73 genome, although the sequenced B73 variety is phenotypically *ga2*. If they indeed represent the B73 haplotype of the *ga2* locus, these genes are predicted to be non-functional alleles of *Ga2-f* and *Ga2-m*.

In *Zea* CI systems, unlike SI systems, there is not a large diversity of alleles for any one of the loci, but perhaps the diversity is seen in comparing different loci, and the number of distinct functional types of PME38-like and QRT1-like CI protein pairs may be expanded through surveys of other species. In searching for other PME-based CI systems in maize or other species, there are several criteria that are predicted to be critical for a CI system. First, the expression level of the female/PME38-like gene should be very high in the pistil for rapid inhibition of incompatible pollen grains. This is the case for both *Tcb1-f* and *Ga1-f* (Lu et al. 2019; Moran Lauter et al. 2017). Second, the expression of the male/*QRT1-like* gene should be very high in the pollen so as to overcome the activity of the barrier in the pistil (as is the case for *Ga1-m* (Zhang et al. 2018) and our unpublished observations on a different *Ga1-m* allele from teosinte). Finally, for the system to maintain cross-incompatibility function, the female-male gene pair must be very tightly linked genetically or the system will break down via recombination. Preliminary evidence suggests that such an adjacent *PME38-like QRT1-like* gene pair is present in the sorghum genome, although their expression patterns and allelic variability within sorghum have not been analyzed yet.

If the male and female factors of a CI system can segregate independently, the model proposed here suggests that several types of reproductive systems can occur within a population when only one CI system is involved, including: individuals that are cross-incompatible with each other (one has male and female functions and one has neither); individuals that are self-incompatible (if they carry the female function but not the matching male function); and self-incompatibility conditioned by these systems if the female functions are linked in *cis* with a non-matching male function and the matching male function is linked in repulsion (in *cis* with a different female allele). Exactly, how many different types of these matching pairs are possible (and hence how many genetically isolated populations are possible) is currently unknown, but it is likely that at least three mostly cross-incompatible PME-systems are present in *Zea*. These

CI systems have already been used extensively for breeding programs, particularly for popcorn varieties. However, their continued utility may be compromised as more tropical maize germplasm, some of which contain *Ga1-m* or *Ga2-m*, are incorporated into other breeding programs (Kermicle 2006; Jones and Goodman 2018; Kermicle and Evans 2010; Kermicle et al. 2006). Stacking multiple CI loci in the same line will help to mitigate some of these issues, but a search for additional CI-conferring PME male–female gene pairs in other grasses may identify CI systems that could be transferred into maize and function independently of the three existing systems.

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Compliance with ethical standards

Conflicts of interests Author MMS Evans is named as inventor on U.S. Patent No. 7,074,984: Cross-incompatibility traits from teosinte and their use in corn. Jerry L. Kermicle, Steven R. Gerrish, and Matthew M. Evans. The other authors declare no competing interests.

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