Mechanism of Self-Sterility in a Hermaphroditic Chordate

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Mechanism of Self-Sterility in a Hermaphroditic Chordate

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Hermaphroditic organisms avoid inbreeding by a system of self-incompatibility (SI). A primitive chordate (ascidian) *Ciona intestinalis* is an example of such an organism, but the molecular mechanism underlying its SI system is not known. Here, we show that the SI system is governed by two gene loci that act cooperatively. Each locus contains a tightly linked pair of polycystin 1–related receptor (*s-Themis*) and fibrinogen-like ligand (*v-Themis*) genes, the latter of which is located in the first intron of *s-Themis* but transcribed in the opposite direction. These genes may encode male- and female-side self-recognition molecules. The SI system of *C. intestinalis* has a similar framework to that of flowering plants but utilizing different molecules.

Self-incompatibility (SI) is a system found in many hermaphroditic organisms, including flowering plants, which prevents self-fertilization and promotes outcrossing (*1*). Fertilization strategies based on the recognition of self or nonself have been reported (*2*, *3*). Known SI systems include multiple proteins that are encoded within a specific gene locus termed the SI specificity–determining locus. The self-recognition, or allorecognition, process requires a male factor and a female factor, and both of these factors must cosegregate chromosomally to enable successful inheritance of the allospecific interaction. Tight genetic linkage of the male and female SI genes is a common key feature in the previously characterized plant SI systems (*4*).

The invertebrate chordate *Ciona intestinalis* has a highly evolved SI system. Interest in the ascidian SI system dates back to the works of Thomas Hunt Morgan (*5*–*8*). Quite a bit is known about the *Ciona* SI system: (i) A self/nonself-discrimination site resides on the egg's vitelline coat (VC), which is an acellular matrix surrounding the egg (*9*). The VC shows higher affinity to the nonself sperm than to the self sperm. Fertility of sperm is closely related to its capability to bind to the VC (*9*, *10*). This suggests that the male SI factor is expressed on the sperm surface. (ii) Acquisition of self-sterility takes place during oocyte maturation (*11*). (iii) The barrier against self-fertilization is abolished by treatment with mildly acidic seawater or by addition of proteases (*5*). When treating eggs with weak acid to allow self-fertilization (Fig. 1A), Morgan observed two types of cross-sterility, which is nonself sterility between gametes from different animals, within the obtained self-fertilized F_1 siblings (*6*, *7*): one-way cross-sterility and bidirectional cross-sterility. In the case of one-way cross-sterility, the eggs are fertilized by nonself-sperm, but no fertilization occurs in the opposite gamete combination. On the other hand, no fertilization is observed in both gamete combinations in bidirectional cross-sterility. Occurrence of one-way cross-sterility is unlikely to be due to a crossover between the male and female SI genes, which would soon disrupt the SI system during a few generations. Instead, Morgan introduced a "haploid sperm hypothesis" to explain this phenomenon, which proposes that SI specificity is determined by haploid expression in sperm but by diploid expression in eggs (Fig. 1B) (*6*–*8*). According to this hypothesis, a parent heterozygous at the SI locus (represented as A/a) produces two populations of sperm (A-sperm and a-sperm), either of which can fertilize two types of homozygous eggs (A/A and a/a). In contrast, sperm (A-sperm and a-sperm) from two types of homozygotes (A/A and a/a) are unable to fertilize heterozygous eggs (A/a), because heterozygote eggs have (in the VC) both types of female SI gene products.

We repeated Morgan's self-fertilization experiment (Fig. 1A) (*12*) and obtained several batches of F_1 populations, among which were two batches (H and G) that we analyzed in detail and obtained noncontradictory results (figs. S1 and S2). Batch H contained 23 self-fertilized siblings from a single parent. We examined the pairwise sterility or fertility of gametes from these 23 animals and one nonsibling animal as a control in all the 576 ($= 24 \times 24$) combinations (fig. S1A). According to the pattern of cross-sterility, 23 selfed F_1 siblings were clustered into six distinct groups (fig. S1B). Gametes were bidirectionally sterile within each group, but reciprocal fertility and one-way sterility were observed between different groups. This grouping agreed well with that predicted from an expanded version of the above-mentioned genetic scheme based on the "haploid sperm hypothesis" to two SI loci (designated as locus A and B) system (Fig. 1C, also see fig. S1B).

We then undertook the positional cloning of these two loci by searching for genetic markers that show a similar segregation pattern as that predicted from the above genetic scheme. Genetic mapping using the H batch revealed that loci A and B reside in chromosomes 2q and 7q, respectively (tables S1 and S2). Fine mapping was followed utilizing the G and H batches, and the candidate regions of loci A and B were finally restricted to as narrow as 170 kilobase pairs and 1 megabase pair long, respectively (fig. S3). Locus A appeared to contain only about 20 transcription units (table S3). No overall synteny between these two loci was observed with two exceptions.

Synteny was observed for a homolog of the mammalian *PKD1* (*polycystin-1*) gene, a causative gene for autosomal dominant polycystic kidney disease (ADPKD), which was found in both loci (Fig. 2A and figs. S4 and S5). We designated these two PKD1 homologs as *s* (sperm)-*Themis-A* (in locus A) and *-B* (in locus B), respectively. (Themis is a Greek goddess who is the embodiment of divine order, law, and custom and prohibits incest.) PKD1 is a transmembrane receptor and interacts with PKD2 (polycystin-2), which is a product of another causative gene for ADPKD and structurally related to PKD1, to act as a calcium-permeable cation channel complex (*13*, *14*). It is thought that in this complex PKD1 plays a role to modulate the ion conductance of the PKD2 channel (*15*). PKD1 family members of other organisms are known to play key roles in fertilization. For example, in sea urchin sperm, the acrosome reaction requires activation of two PKD1 orthologs (REJ1 and REJ3, receptor for egg jelly) (*16*, *17*). REJ3 is shown to bind physically to the sea urchin PKD2 ortholog (suPC2) in the plasma membrane over the acrosomal vesicle (*18*). Also, in flies, there may be involvement of PKD1 members in the gamete interaction process because disruption of the *PKD2* gene results in male sterility without affecting spermatogenesis (*19*). PKD1 family members usually contain 11 transmembrane domains, and s-Themis-B also contains these putative 11 domains. In contrast, s-Themis-A is a diverse member and contains five transmembrane domains. Both versions of s-Themis are polymorphic in the N-terminal hypervariable regions (fig. S6, A and B; see supporting online text). For example, only 29% of amino acids (42% of nucleotides) were identical between two alleles of s-Themis-B shown in fig. S6B, whereas it is reported that genome sequences derived from two homologous chromosomes in *C. intestinalis* show more than 99% identity to each other in coding regions at the nucleotide level (*20*). A and B s-Themis are relatively conserved in the C-terminal side of the REJ module and contain a signal peptide, an N-terminal hypervariable region, an REJ module, and a heterotrimeric GTP–binding protein (G protein)–coupled receptor proteolytic site (GPS) domain in the extracellular region and a lipoxygenase homology domain in the cytoplasmic region (Fig. 2A and figs. S4 and S5). Transcripts of *s-Themis-A* and *-B* were found in a testis cDNA library (*21*) (also see table S3), which suggested that these gene products are on spermatozoa.

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The other gene shared by both loci was a fibrinogen-like gene (Fig. 2A and figs. S4 and S5). In parallel with this genetic study, we are currently identifying VC components in this species by mass spectrometry–based proteomic analysis. Among about 20 proteins encoded in the candidate region for locus A, the fibrinogen-like molecule turned out to be the only one included in the identified VC components (table S3; figs. S6C and S7). Similarly, another fibrinogen-like protein encoded in the candidate region for locus B was also found in the VC (figs. S6D and S7). We thus designated these genes as *v* (vitelline coat)-*Themis-A* and -*B*. Both v-Themis proteins were relatively small, consisting of a single fibrinogen-α/β/γ-chain C-terminal globular domain, but extremely polymorphic (fig. S6, C and D; see supporting online text). This domain is known to be responsible for the self/nonself (pathogen)–discrimination in the innate immunity system of a horseshoe crab (*22*). The *v-Themis* genes locate within the long first intron of respective *s-Themis* genes, which are transcribed in the opposite direction (Fig. 2B and figs. S4 and S5). The hypervariable regions of *s-Themis* and *v-Themis* were adjoining in both loci.

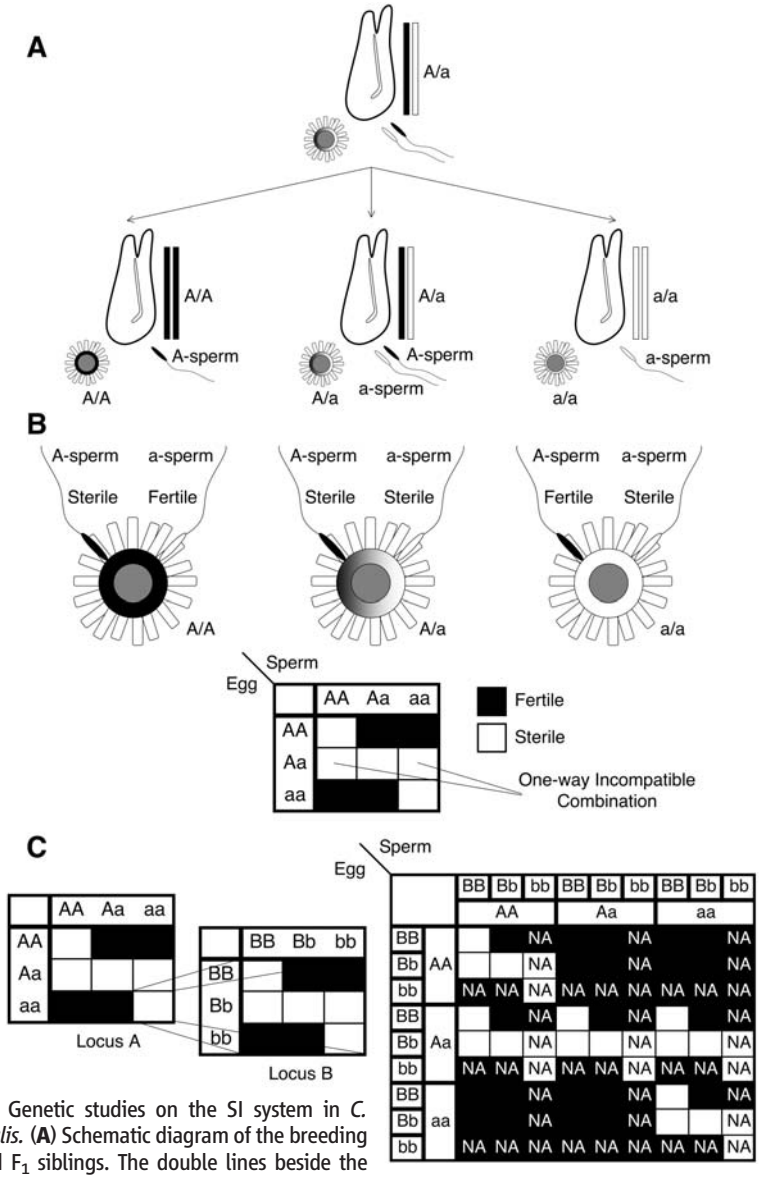

Fig. 1. Genetic studies on the SI system in *C. intestinalis.* (**A**) Schematic diagram of the breeding of selfed F_1 siblings. The double lines beside the animals represent the diploid hypothetical SI gene. Artificial self-fertilization of an animal, which is heterozygous in the gene concerned, produces three types of F_1 siblings (two types of homozygotes and one heterozygote). (**B**) The "haploid sperm hypothesis" originally proposed by Morgan. A predicted fertility or sterility for each combination of genotypes is shown in a (3 × 3) panel (bottom). (**C**) Summary of the pairwise fertility or sterility between the selfed F_1 siblings (batch H) (see fig. S1 for details). The results summarized on the right show a nested pattern of the (3 × 3) units shown in (B), left. This indicates that there are two SI loci in *Ciona* system (shown as A and B), both of which function in the manner shown in (B). It should be noted that a self-recognition signal in either locus A or B is not sufficient to block fertilization, but both signals are necessary for the establishment of sterility. Two alleles in locus A (B) were indicated as A (B) and a (b). The bb homozygotes were absent in the 23 siblings investigated (because of possible impairment in development or growth) and are indicated as NA (not available).

Taking these findings together, we propose that SI is controlled by two loci in *C. intestinalis*, each of which contains a male determinant *s-Themis*, and a female determinant *v-Themis* (Fig. 2C). *s-Themis* and *v-Themis* may have many different alleles, and these genes comprise a single haplotype because of their extreme genetic proximity. We propose that s-Themis factors act as receptors that can specifically interact with the corresponding v-Themis encoded in the same haplotype. It is possible that when these two s-Themis receptors recognize respective autologous v-Themis ligands as self on the VC, a sperm regards the egg as self (deduction from the genetic scheme shown in Fig. 1C) and actively weakens its binding ability to the VC in order to detach. Previous cytological observations show that self sperm can bind well to the autologous VC initially, but it will detach within a few minutes (*9*, *10*). We speculate that such duration reflects the time consumed by sperm to execute a series of self-recognition reactions. *Ciona savignyi*, a close relative of *C. intestinalis*, is reported to be a self-fertile species; however, self/nonself-discrimination still takes place in this species, resulting in a longer time requirement for self-fertilization than for cross-fertilization (*23*). The PKD1 signal is known to induce the elevation of cytoplasmic [Ca^{2+}] via the PKD2 channel (*24*). It is possible that the elevation of [Ca^{2+}] in the sperm cytoplasm may be involved in these processes.

Histocompatibility in the colonial ascidian, *Botryllus schlosseri*, is controlled by a single highly polymorphic locus, *Fu/HC*. Candidates for *Fu/HC* genes (*cFu/HC*, *fester*) have been recently discovered (*25*, *26*). *cFu/HC* encodes an immunoglobulin superfamily member containing epidermal growth factor (EGF)–like repeats, whereas *fester* is a Sushi domain–containing gene. Sushi domains are also found in sp56, a mouse sperm protein thought to be involved in binding sperm to the egg zona pellucida (*27*). Allorecognition involved in the *Botryllus* histocompatibility may be more complicated than that in the *Ciona* SI system because the former includes an acquired immunity–like mechanism mediated by individual specific splice variants of *fester* (*26*). In another solitary ascidian *Halocynthia roretzi*, we previously reported a 70-kD VC protein, HrVC70, consisting of 12 EGF-like repeats, as a candidate for the female SI factor (*28*). Possible involvement of homologs of *s-Themis*–*v-Themis* in the SI system of *H. roretzi* remains to be determined.

In flowering plants, such as Brassicaceae, pollen expresses S-locus cysteine-rich protein (SCR), a ligand for an S-locus receptor kinase (SRK) that is expressed in stigma, and both multiallelic genes are located in an *S-locus*,

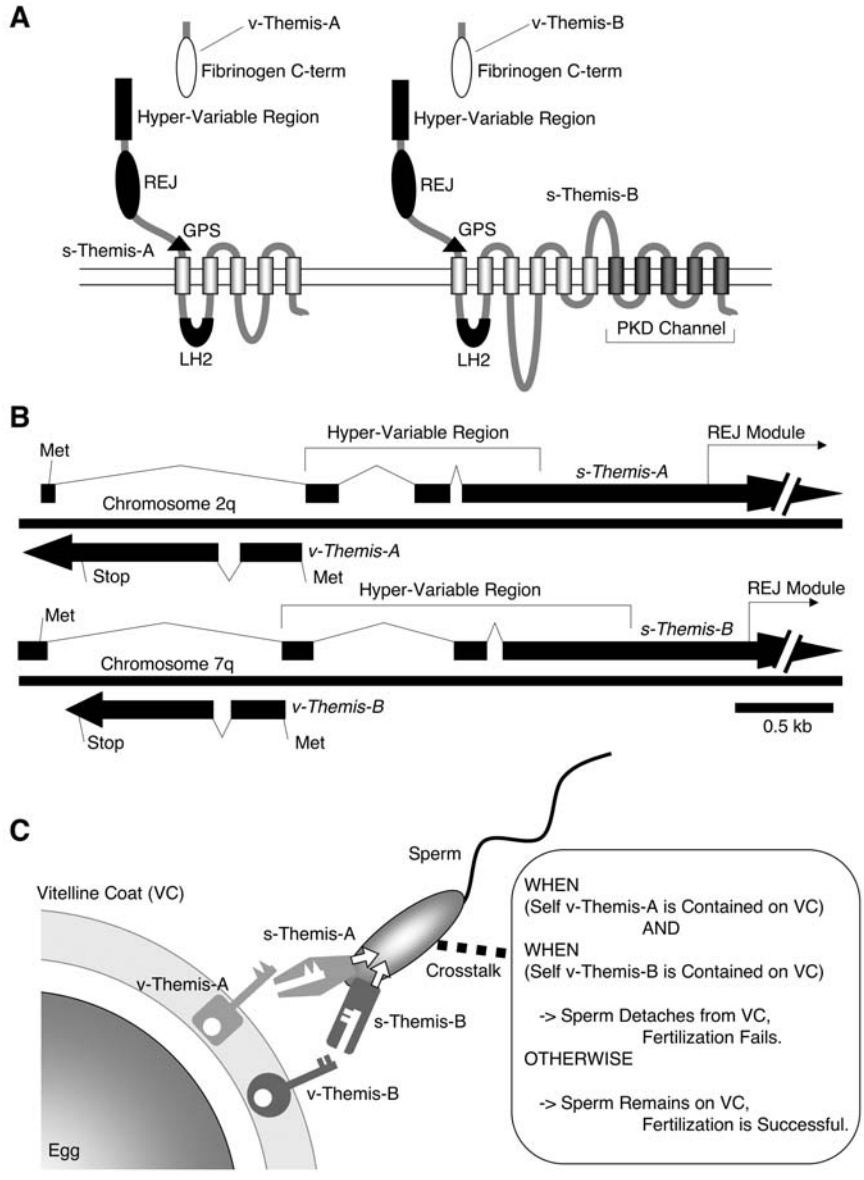

Fig. 2. Molecular mechanism of the SI system. (**A**) Structures of s-Themis-A, v-Themis-A, s-Themis-B, and v-Themis-B. s-Themis-A and -B are 5-pass and 11-pass transmembrane proteins, respectively. REJ, REJ module; GPS, G protein–coupled receptor proteolytic site; LH2, lipoxygenase homology domain–2; fibrinogen C-term, fibrinogen α/β/γ-chain C-terminal globular domain. (**B**) Schematic genomic structure of *v-Themis-A* and 5′-terminal region of *s-Themis-A* on chromosome 2q, and of *v-Themis-B* and 5′-terminal region of *s-Themis-B* on chromosome 7q (see figs. S4 and S5). *v-Themis* locates within the first intron of the respective *s-Themis*. Met, predicted translation initiation site; Stop, translation termination site. (**C**) A model of s-Themis/v-Themis–mediated SI system. We propose that when both s-Themis-A and -B ("key holes") on the sperm surface recognize respective v-Themis ("keys") on the VC as self, sperm detaches. Otherwise, sperm remains on the VC and then penetrates through the VC to fertilize the egg.

which is inherited as one segregating unit (*1*). It is known that a specific interaction between SCR and SRK in the same haplotype triggers SRK activation, which inhibits pollen tube elongation in stigma and prevents self-fertilization (*1*). Taking these systems into account, we note that a framework of an animal SI system observed in *C. intestinalis* is strikingly similar to those of flowering plants, although they utilize different molecules in their self-recognition strategies.

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Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 to S7
Tables S1 to S3
References

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