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Report

Gamete Attachment Requires GEX2 for Successful Fertilization in *Arabidopsis*

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Summary

Fertilization requires recognition, attachment, and membrane fusion between gametes. In metazoans, rapidly evolving surface proteins contribute to gamete recognition and adhesion [1]. Flowering plants evolved a double fertilization process wherein two immotile sperm cells are delivered to female gametes by the pollen tube, guided by elaborate communications between male and female reproductive organs [2–7]. Once released, the sperm cells contact female gametes directly prior to gamete fusion. It remains unclear whether active gamete recognition and attachment mechanisms are required for double fertilization. Here, we provide functional characterization of *Arabidopsis GAMETE EXPRESSED 2* (*GEX2*), which encodes a sperm-expressed protein of unknown function [8]. GEX2 is localized to the sperm membrane and contains extracellular immunoglobulin-like domains, similar to gamete interaction factors in algae and mammals [9, 10]. Using a new in vivo assay, we demonstrate that GEX2 is required for gamete attachment, in the absence of which double fertilization is compromised. Ka/Ks analyses indicate relatively rapid evolution of GEX2, like other proteins involved in male and female interactions [1, 3]. We conclude that surface proteins involved in gamete attachment and recognition exist in plants with immotile gametes, similar to algae and metazoans [11, 12]. This conservation broadens the repertoire of research for plant reproduction factors to mechanisms demonstrated in animals.

Results and Discussion

Isolation of a Mutant Defective for Gamete Fusion

We mutagenized a double marker (DM) line, in which sperm nuclei and the central cell are labeled with HTR10-RFP (snRFP) and ΔFWA-GFP (ccGFP), respectively [13, 14], and screened for plants defective for double fertilization. As a result, we isolated a mutant line, Y47, showing one set of unfused sperm cells (Figure 1A). Two sets of unfused sperm pairs were rarely detected, likely delivered by a second pollen tube as reported for other mutants defective in sperm release [15] (see Figure S1A available online). In the Y47 line, aniline blue-stained pollen tubes reached all ovules normally regardless of whether they showed seed development or not, and they did not show any coiled unruptured invading pollen tubes typical of *feronia*-class mutants [16, 17] (Figures S1B and S1C). Time-lapse imaging showed that in contrast to wild-type (WT) (Figure 1B), the Y47 sperm pair arrests after reaching the central cell (Figure 1C; Movie S1). We concluded that Y47 sterility is caused by deficiency in gamete recognition, adhesion, or fusion. The Y47 phenotype was detected only in self-pollinated heterozygous Y47 plants and in crosses between WT ovules and Y47 mutant pollen, in contrast to WT self and crosses between WT pollen and Y47 mutant ovules (Figure 1D). In addition, crosses between WT ovules and Y47 homozygous mutant pollen produced a percentage of unfertilized ovules approximately 2-fold higher than a similar cross with the heterozygous mutant (Figure 1D), further supporting that Y47 causes a male gametophytic defect of fertilization.

GEX2 Loss of Function Is Defective for Fertilization

Genetic mapping identified a 271 kb domain in chromosome 5 that contained the mutation responsible for the Y47 male sterility. Because this domain contains a sperm-specific gene candidate, *GAMETE EXPRESSED 2* (*GEX2*) (At5g49150) [8], we sequenced the genomic *GEX2* and *GEX2* cDNA isolated from WT and Y47 plants. A point mutation exchanging G to A was detected in the first base of the eighth intron of *GEX2* (Figures 2A and S1D). The mutation was also confirmed in a cleaved amplified polymorphic sequence (CAPS) assay (Figure S1D). As a result, the Y47 line was named *gex2-1*. *gex2-1* affects the consensus 5′ GT sequence involved in intron splicing [18] and causes production of a longer PCR product containing the unspliced intron (Figure 2B). Missplicing of *gex2-1* transcripts results in a frameshift leading to a stop codon in the ninth exon (Figure S1D), questioning whether *gex2-1* is a null allele. We isolated another *Arabidopsis GEX2* mutant line (FLAG_441D08), in which the *GEX2* gene is disrupted by T-DNA insertion (Figure 2A), preventing production of functional *GEX2* transcripts in homozygous FLAG_441D08 plants (Figure 2B). This line, named *gex2-2*, showed the same defects observed in *gex2-1* (Figure 2C), suggesting that both *gex2-1* and *gex2-2* are null alleles that produce sperm cells unable to perform double fertilization.

To confirm that the loss of *GEX2* was responsible for the male sterility phenotype, we produced a homozygous *gex2-1* line expressing genomic *GEX2* conjugated with *GFP* cDNA, under the *GEX2* promoter ($-/gex2\text{-}1^{+/gGEX2\text{-}GFP}$). Fertility was restored in complemented mutant plants (Figure S1E), showing that *GEX2* is required for functional sperm cells and that *gex2-1* is a recessive mutation.

GEX2 Is Located at the Plasma Membrane of Sperm Cells

GEX2 was initially described as composed of 12 exons and encoding 885 amino acid residues [8] (Figure 2A). However, we

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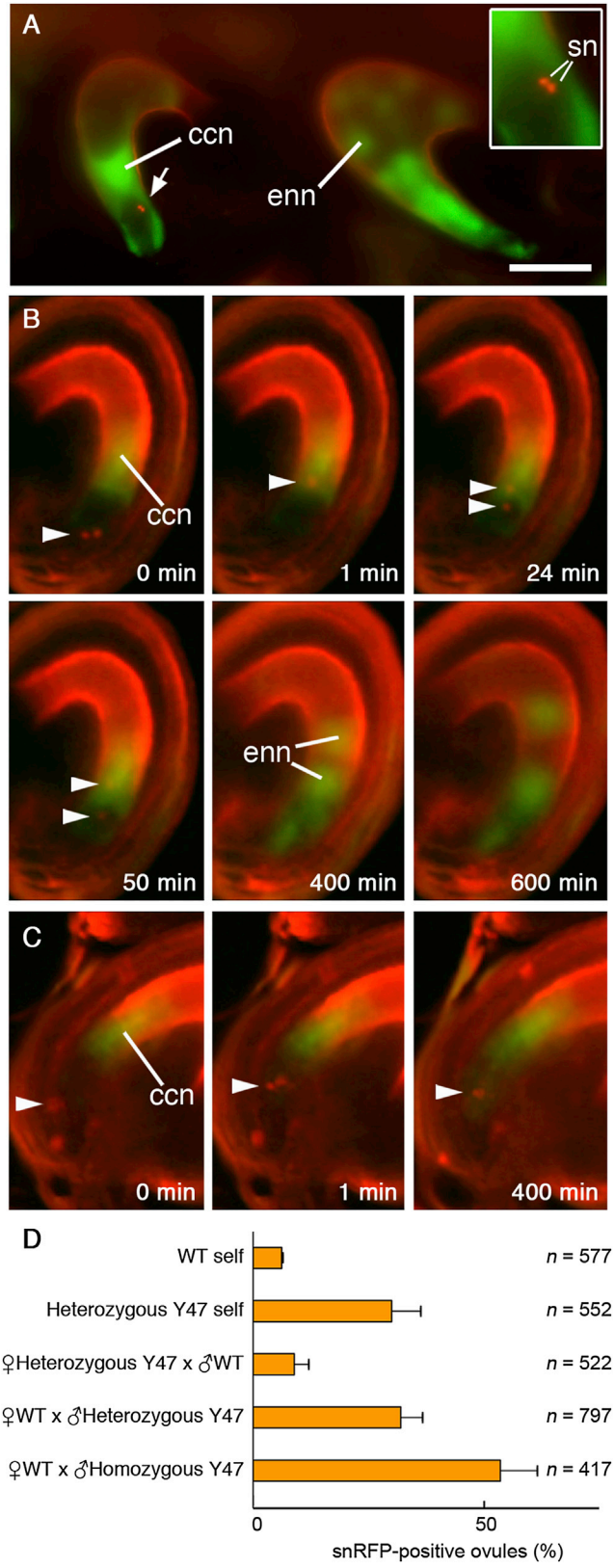

Figure 1. Identification of the New Gamete Fusion-Defective Mutant Y47

(A) A gamete fusion-defective line, Y47, occasionally showed sperm nucleus (sn) signals from unfused sperm cells in ovules (arrow). The area indicated by the arrow is magnified in the inset. In most ovules showing an snRFP signal pair, no morphological changes occurred with the central cell nucleus (ccn) (left ovule), while proliferating endosperm nuclei (enn) were detected in ovules with no snRFP signals (right ovule). The scale bar represents 50 μm.

(B and C) Time-lapse imaging of normal and impaired double fertilization in live *Arabidopsis* reproductive tissues.

(B) In WT plants, one of the sperm nuclei initiated karyogamy with the central cell nucleus within 1 hr after sperm entering the embryo sac (upper arrowhead in the third and fourth panels of B), and the first karyokinesis of endosperm nuclei took place within 5 hr after completion of karyogamy (the fifth and last panels of B).

(C) In contrast, Y47 sperm nuclei (arrowhead) remained unfused even at more than 6 hr after sperm entrance (right panel).

(D) Ovules showing an snRFP signal were counted. Three to six siliques at 1 day after pollination (DAP) were collected from two to three individuals for each crossing group.

detected extended sequences in both the 5′ and 3′ directions of the *GEX2* cDNA. As a result, we found that the *GEX2* comprises 16 exons and encodes 1,087 residues (Figure 2A). Domain prediction programs predicted that *GEX2* encodes a single-pass transmembrane (TM) protein composed of an N-terminal signal sequence (SS), filamin repeat domains (FLMN), and a C-terminal TM domain (Figure 2D). In *gex2-1* pollen expressing *pGEX2::gGEX2-GFP* (Figures 2E–2G), GEX2-GFP was detected specifically in the plasma membrane of sperm cells, resembling the localization of GCS1 [19–22] (Figures 2F, 2H, and 2I). In onion epidermal cells, both GEX2-GFP and GCS1-GFP, expressed under the control of the cauliflower mosaic virus 35S promoter, were detected in plasma membranes (Figures S2A–S2F), suggesting that GEX2 localization to the plasma membrane does not require sperm cell-specific factors.

gex2 Causes Single Fertilization and Seed Abortion

In addition to undeveloped ovules, aborted seeds were detected at a notable frequency (~20%) in selfed *gex2* mutants (Figure 3A) and when *gex2* mutants were used as pollen donors (Figure 3B), but not in *−/gex2-1* plants expressing *pGEX2::gGEX2-GFP* (Figure S1E). We observed that failure of a single sperm cell fusion led to seeds that contain either an embryo or an endosperm (Figure 3C). To further confirm the linkage between seed abortion and *gex2* sperm phenotype, we combined genomic *GEX2* or *gex2-1* with *snGFP* and introduced the constructs (*snGFP;gGEX2* or *snGFP;ggex2-1*, respectively) into *−/gex2-2* plants (Figure S3A). After pollination of WT ovules with $-/gex2\text{-}2^{+/snGFP;gGEX2}$ pollen, the seed sets derived from this outcross were almost normal, suggesting that the genomic *GEX2* fragment complemented the *gex2-2* phenotype (Figure S3B). In contrast, outcross between $-/gex2\text{-}2^{+/snGFP;ggex2\text{-}1}$ (male) and WT (female) plants led to seed arrest (Figure S3B), in which unfused sperm cells were frequently detected (Figures S3C and S3D). We conclude that in the absence of GEX2, sperm cells are delivered correctly but are unable to interact properly with female gametes in such a manner that fertilization is prevented with both or a single female gamete.

GEX2 Is Required for Gamete Attachment

We tested whether GEX2 is involved in gamete attachment. In *Arabidopsis*, the gametes are tightly arranged with companion cells in the embryo sac, preventing a direct assessment of the attachment between sperm cells and female gametes. To enable evaluation of the degree of adhesion between gametes, we treated ovules expressing egg membrane and nucleus markers (*emGFP* (*pDD45::GFP-PIP2a* [22]) and *enRFP*, respectively) or a central cell marker (*ccGFP*) with polysaccharide-digesting enzymes. In digested embryo sacs, egg and

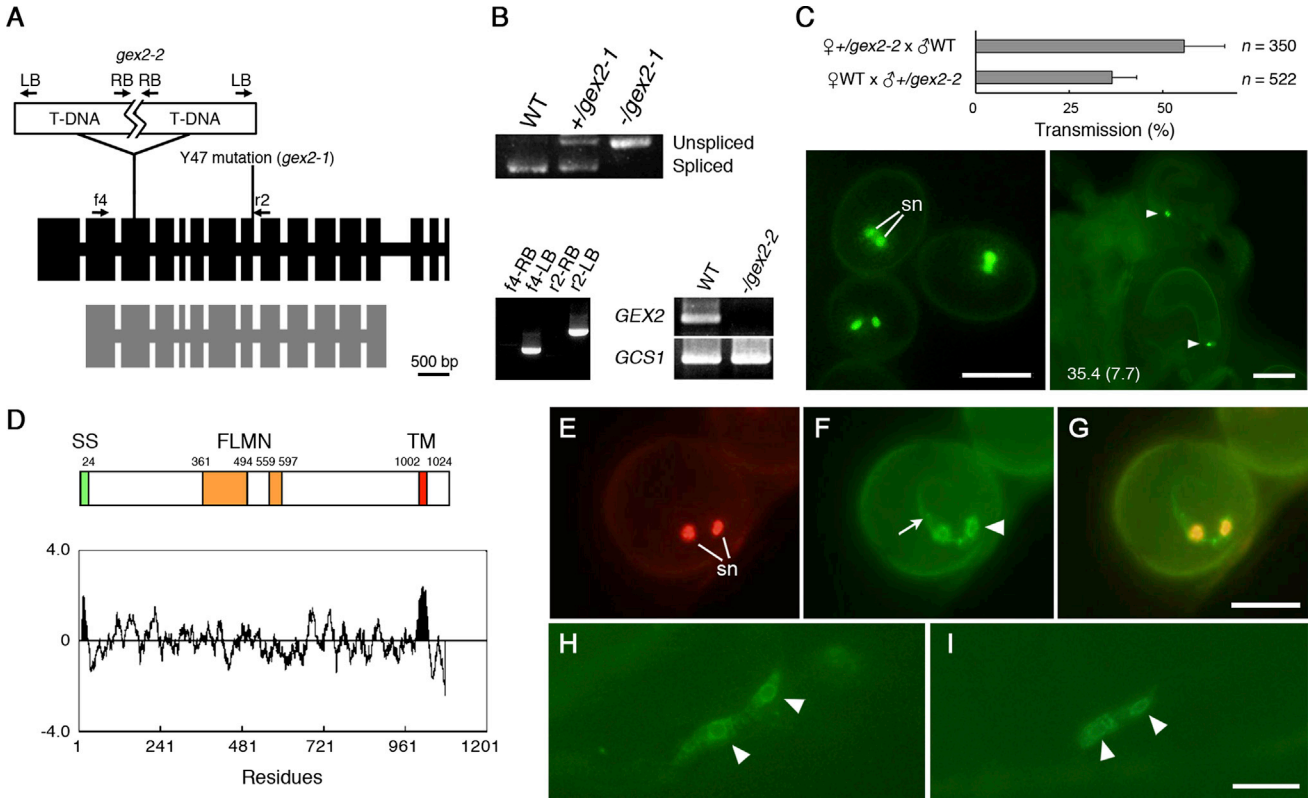

Figure 2. Detection of Mutations Causing Male Sterility in *gex2* Mutants and Characterization of GEX2 Structure

(A) Gene structure of *Arabidopsis GEX2*. Exons and introns are represented with vertical boxes and horizontal lines, respectively. The newly determined *GEX2* structure in this study is drawn in black, while that of the gene previously reported by Engel et al. is shown in gray [8]. The position of the point mutation in the Y47 line is indicated. In the *gex2-2* line, at least two copies of inverted T-DNA repeat are found in the third exon. The arrows represent primer positions used for T-DNA detection.

(B) PCR assays to detect *gex2* mutations. Because a point mutation in *gex2-1* lines prevents proper mRNA splicing, longer transcripts were detected in both heterozygous and homozygous *gex2-1* flowers (top panel). Only two sets of primers, f4-LB and r2-LB, detected the T-DNA fragments in −/*gex2-2* plants (bottom left panel). In RT-PCR assays, no functional *GEX2* expression was detected, whereas *GCS1* expression was obvious in −/*gex2-2* flowers (bottom right panel).

(C) Phenotypes of *gex2-2* plants. *gex2-2* transmission was evaluated with that of the Kan-resistant gene (Kan^R) contained in the T-DNA insert (top panel). Offspring seeds were collected from two to three individuals for each crossing group. $-/gex2\text{-}2^{+/snGFP}$ plants, in which sperm nuclei are GFP labeled, were obtained (bottom left). Frequent snGFP signals were detected in WT ovules after pollination with $-/gex2\text{-}2^{+/snGFP}$ pollen (bottom right). The frequency (percentage) of snGFP-positive ovules is shown with the ±SD in parentheses (n = 333). Scale bars represent 15 μm (bottom left) and 50 μm (bottom right).

(D) Characterization of the GEX2 protein. GEX2 protein is composed of an N-terminal signal sequence (SS), filamin repeat domains (FLMN), and a C-terminal transmembrane domain (top). Amino acid positions of those domains above are indicated. The hydropathy profile of GEX2 detected remarkable peaks (shaded black) in the N- and C-terminal regions, reflecting the SS and TM domains, respectively (bottom).

(E–G) *pGEX2::gGEX2-GFP* expression in −/*gex2-1* pollen. (E) and (F) are an identical field group and are merged in (G). Sperm nuclei (sn) are specifically labeled with HTR10-RFP (E). The GEX2-GFP signals were detected specifically in sperm cells (F). Tailed GFP signals (arrow) were observed besides sn-surrounding ones (arrowhead). Scale bar represents 10 μm.

(H and I) GEX2-GFP signals were also detected in sperm cells migrating in a pollen tube of the GEX2-GFP-expressing line (H, arrowheads). Similar sn-surrounding signals, derived from endomembrane, were also detected in a GCS1-GFP-expressing line (I, arrowheads). Scale bar represents 10 μm.

central cells became round (Figures 4A and 4E), enabling us to assess the degree of attachment between the egg/central cells and sperm cells in ovules after pollination with pollen from −/*gex2-1* or +/*gcs1* mutants expressing *snRFP*. When pollen was provided by +/*gcs1* plants, plasmolyzed ovules frequently showed a pair of sperm cells attached to the egg cell (Figure 4B) or the central cell (Figure 4F). In contrast, when pollen was provided by −/*gex2-1*, we observed predominance of two types of plasmolyzed ovules with (1) one attached sperm cell and one detached sperm cell (Figures 4C and 4G) and (2) two detached sperm cells (Figures 4D and 4H). Sperm cell detachment in −/*gex2-1* was also detected in ovules without enzymatic treatment, indicating that the gamete detachment is due not to polysaccharide digestion but to *gex2* (Figures S4A–S4C). In both groups, only one snRFP signal instead of two was frequently detected and likely corresponded to single fertilization events or sperm cell death (insets in Figures 4C, 4D, 4G, and 4H). The percentage of ovules containing no attached sperm cells was greater in *gex2-1* than in *gcs1* mutants (Figures 4D, 4H, and 4I). These results suggest that GEX2 contributes to gamete attachment, ensuring stable double fertilization.

In metazoans, rapid divergence of genes encoding recognition and attachment factors participates in reproductive isolation [1]. We analyzed molecular divergence in GEX2 and GCS1 between the closely related species *A. thaliana* and *A. lyrata*.

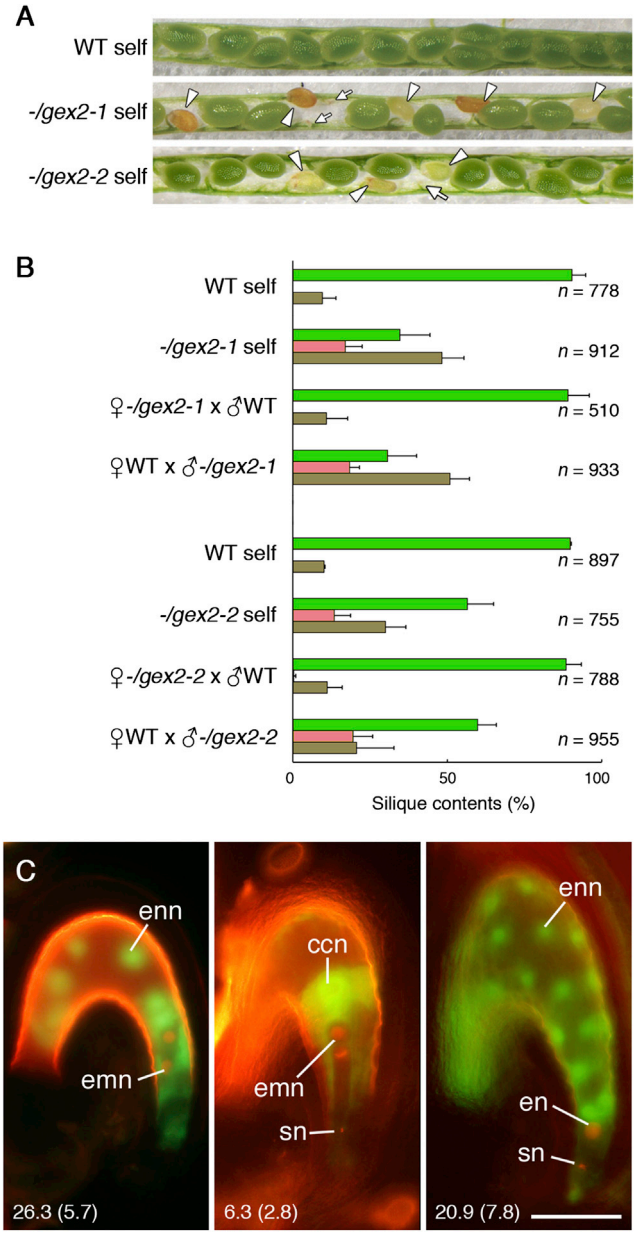

Figure 3. Abnormal Seed Development in *gex2* Plants

(A) Almost all seeds showed normal development in selfed WT plants, while undeveloped ovules (arrows) and aborted seeds (arrowheads) were frequently detected in −/*gex2-1* and −/*gex2-2* plants.

(B) Normally developing seeds (green), aborted seeds (pink), and undeveloped ovules (brown) were counted. Three to five siliques were collected from each of three individuals at ~10 DAP for each crossing group.

(C) Various fertilization patterns in the *gex2-1* line. Ovules at 2 DAP were observed and categorized as follows: normal development showing proliferation of both endosperm (enn) and embryo (emn) nuclei (left), development of the embryo alone (center) or the endosperm alone (right), and no development (not shown). The remnants of the snRFP signal were occasionally detected in the incompletely developing ovules described above (sn). The percentage of each development type observed in three individuals (except for "no development") is shown with the ±SD in parentheses (n = 345). Scale bar represents 50 μm.

As an indication of their divergence, the ratio between the number of nonsynonymous substitutions per nonsynonymous site (Ka) and the number of synonymous substitutions per synonymous site (Ks) was calculated (Ka/Ks ratio), based on a sliding window method [3, 23]. Higher Ka/Ks values were detected in *GEX2* than in *GCS1*, suggesting that GEX2 evolves more rapidly than GCS1 (Figure 4J) in accord with their respective functions in recognition/attachment and in homotypic fusion.

Conclusions

GEX2 is expressed in the plasma membrane of sperm cells, and this is also supported by the appearance of the TM domain in our revised annotation of the *GEX2* gene. The expression of both *GEX2* and *GCS1* in sperm cells requires the transcription factor DUO1 [24]. Although a previous study reported that GEX2 is also expressed in the egg cell [25], we were unable to confirm egg cell GEX2 localization, and *gex2* mutation did not affect female fertility, suggesting that GEX2 is not expressed or does not have an essential function in the egg cell.

GEX2 is required for interaction between sperm cells and female gametes, leading to gamete fusion, and our results suggest that although they are no longer motile, higher-plant gametes have retained mechanisms that control gamete recognition and attachment in ancestral organisms with motile gametes. Such interactions do not distinguish the fusion with the egg cell from the central cell, supporting further earlier reports showing that sperm cells have equal capacity to fertilize either female gamete [26, 27].

Flowering plant GEX2 homologs contain at least one filamin-like domain (Figure S4D). Filamin was originally identified as a protein that possesses an N-terminal actin-binding domain and an adjacent rod-shaped arm, composed of filamin repeats, in *Dictyostelium discoideum* (slime mold) and humans [28, 29]. Interestingly, a similar domain is also present in the N terminus of *Chlamydomonas* FUS1, which also functions in gamete attachment [9, 11] (Figure S4D). This suggests that GEX2 and FUS1 might share similar functions in gamete attachment. Filamin domains form immunoglobulin (Ig)-like fold [28, 29]. In mammals, IZUMO1 has been reported as a TM protein critical to gamete fusion, expressed specifically on the sperm surface [10]. IZUMO1 is a member of the Ig superfamily and possesses an Ig domain within its N-terminal sequence [10]. The degree of functional conservation between FUS1, GEX2, and IZUMO1 remains to be established. The lack of complete arrest of fertilization in *gex2* implies that other gamete attachment factors function redundantly with GEX2. Furthermore, the Ka/Ks value between *A. thaliana* and *A. lyrata* GEX2s was highest in the window corresponding to the filamin-like domain, suggesting that divergence in this domain may participate in reproductive isolation (Figure 4J). Our findings not only advance our understanding of mechanisms involved in gamete interaction in plants but also might contribute to improve remediation of fertility defects in crops and allow overcoming sexual reproduction barriers required for breeding new crops.

Accession Numbers

The DNA Data Bank of Japan accession number for the *GEX2* sequence newly determined in this paper is AB743888.

Supplemental Information

Supplemental Information includes four figures, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.11.030.

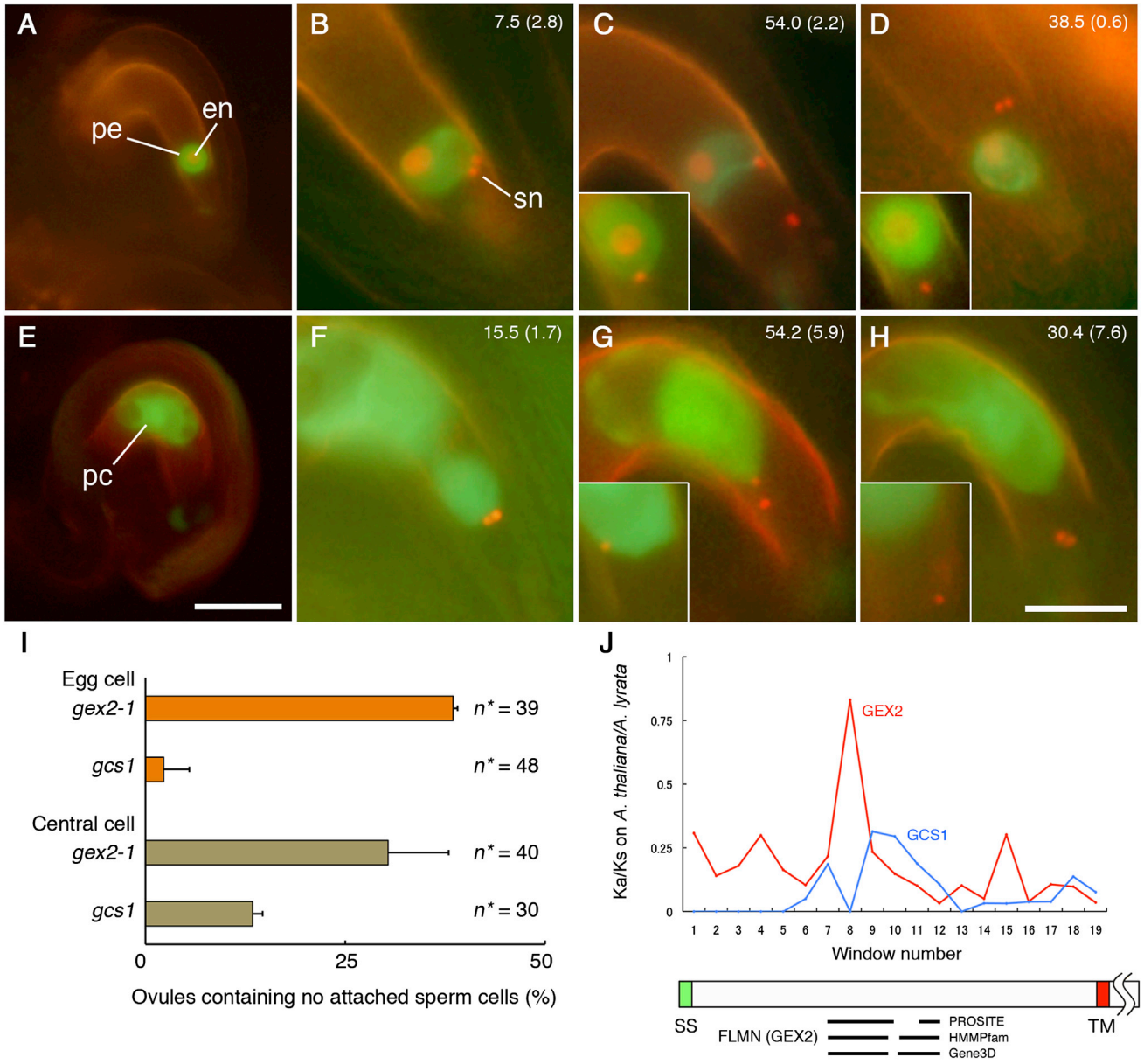

Figure 4. Molecular Function Analyses of GEX2

(A) To assess gamete attachment, we prepared an egg cell marker line expressing both *enRFP* and *emGFP*. After enzymatic treatment, the egg cell (pe) was plasmolyzed and assumed a round shape.

(B–D) When snRFP-positive ovules after pollination with −/*gex2* or +/*gcs1* pollen were observed, unfused sperm cells (sn) attached to (B and C) or detached from (D) the egg cell were detected.

(E) *ccGFP*-expressing ovules were also enzymatically treated to induce plasmolyzed central cells (pc). Scale bar represents 50 μm.

(F–H) Similar sperm behaviors were also detected in *ccGFP*-expressing ovules. Scale bar represents 25 μm. Insets show single sperm cells attached to (C and G) or detached from (D and H) the female gamete. The percentages of each class of phenotype in *gex2-1* are shown with the ±SD in parentheses in (B)–(D) (n = 39) and (F)–(H) (n = 40). Each experiment was repeated two or three times.

(I) Percentage of ovules with detached sperm cells (shown in D and H) in *gex2-1* and *gcs1*. *n** represents the total number of ovules showing completely plasmolyzed egg or central cell and arrested sperm cells.

(J) Ka/Ks ratios on GEX2 and GCS1 were calculated between *A. thaliana* and *A. lyrata* using a sliding window analysis. The window size corresponds to 10% of total length analyzed, and the window slid with a step size of 50% of the window. The averages of Ka/Ks ratio on GEX2 and GCS1 are 0.17 and 0.04, respectively. The positions of FLMNs, deduced by three prediction software tools utilizing their large databases (PROSITE, HMMPfam, and Gene3D; http://www.ebi.ac.uk/Tools/pfa/iprscan/), are shown.

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