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Fertilization: A Sticky Sperm Protein in Plants

During fertilization in eukaryotes, gametes of the opposite sex undergo a complex series of interactions that culminate in cell fusion. A new study on gamete interaction in plants has identified the first protein in multicellular organisms shown by gene disruption to be essential for gamete membrane adhesion.

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Fusion of the plasma membranes of gametes is among the most species-specific events in evolution and is likely an important component of speciation. Fusion of lipid bilayers is not a simple task and the evidence to date indicates that nature has invented membrane fusion only a limited number of times [1]. So many species, so few fusion proteins! Discerning the evolutionary solution to this conundrum has been difficult because of the dearth of proteins shown by gene disruption to be essential for gamete fusion. Only two metazoan proteins have been shown by *in vitro* assays to be involved directly in sperm–egg plasma membrane interactions and by gene disruption to be essential for fertilization: the hydrophobic tetraspanin family member CD9 that is expressed on mouse eggs (and several other cell types) [2–4] and the plasma membrane protein IZUMO1 expressed on mouse sperm [5]. CD9 likely plays a relatively non-specific, facilitative role in fusion, and the step in the membrane fusion reaction that requires IZUMO1 (whose presence is limited to vertebrates) is uncertain [6].

The identification a few years ago of the broadly conserved GENERATIVE CELL SPECIFIC 1/HAPLESS 2 (GCS1/HAP2) family of gamete-specific proteins finally offered a potential solution to the above puzzle. HAP2/GCS1, first reported as a male sterility gene, encodes a sperm protein essential for fertilization in *Arabidopsis* [7,8]. Studies of fertilization in two protists that also express HAP2/GCS1, *Chlamydomonas* and *Plasmodium* demonstrated that gamete membrane adhesion and membrane fusion are distinct molecular events in the membrane fusion reaction. Moreover, it was shown that species specificity during fertilization in the two unicellular organisms derived from species-specific membrane adhesion [9,10]. HAP2/GCS1 is essential for gamete fusion at a step after membrane adhesion in both organisms. Exciting new work published recently in *Current Biology* has now shown that gametes in *hap2/gcs1* mutants of *Arabidopsis* are fully capable of membrane adhesion [11]. Moreover, studies on mutants defective in the sperm protein GEX2 showed that it is involved in membrane adhesion, thus becoming the first protein in multicellular organisms shown by gene disruption to function in gamete interactions before membrane merger, and showed that it indeed undergoes rapid evolution.

In contrast to most metazoans and protists, fertilization in flowering plants (angiosperms) seems almost unduly complicated. First, the sperm are immotile, and must be delivered to the female ovule by the pollen tube. And, plants actually do it in duplicate. Pollen tubes deliver a pair of sperm cells inside each ovule, and each ovule contains two female gametes. The outcome is a double fertilization event. Thus, tightly coordinated gamete interaction mechanisms are required to ensure fusion of one sperm cell with the egg cell and fusion of the second sperm cell with the central cell [12,13]. In spite of this complexity, and in spite of the major challenges to investigating plant gamete interactions *in vitro*, our understanding of cellular and molecular mechanisms of plant fertilization has begun to mature in the past few years, and now surpasses that of metazoans. For example, although HAP2/GCS1 was known to be essential for sperm–egg fusion in *Arabidopsis*, it was not clear how it could function in the membrane fusion reaction, as HAP2/GCS1 in sperm in the pollen tube is sequestered in the endomembrane system and

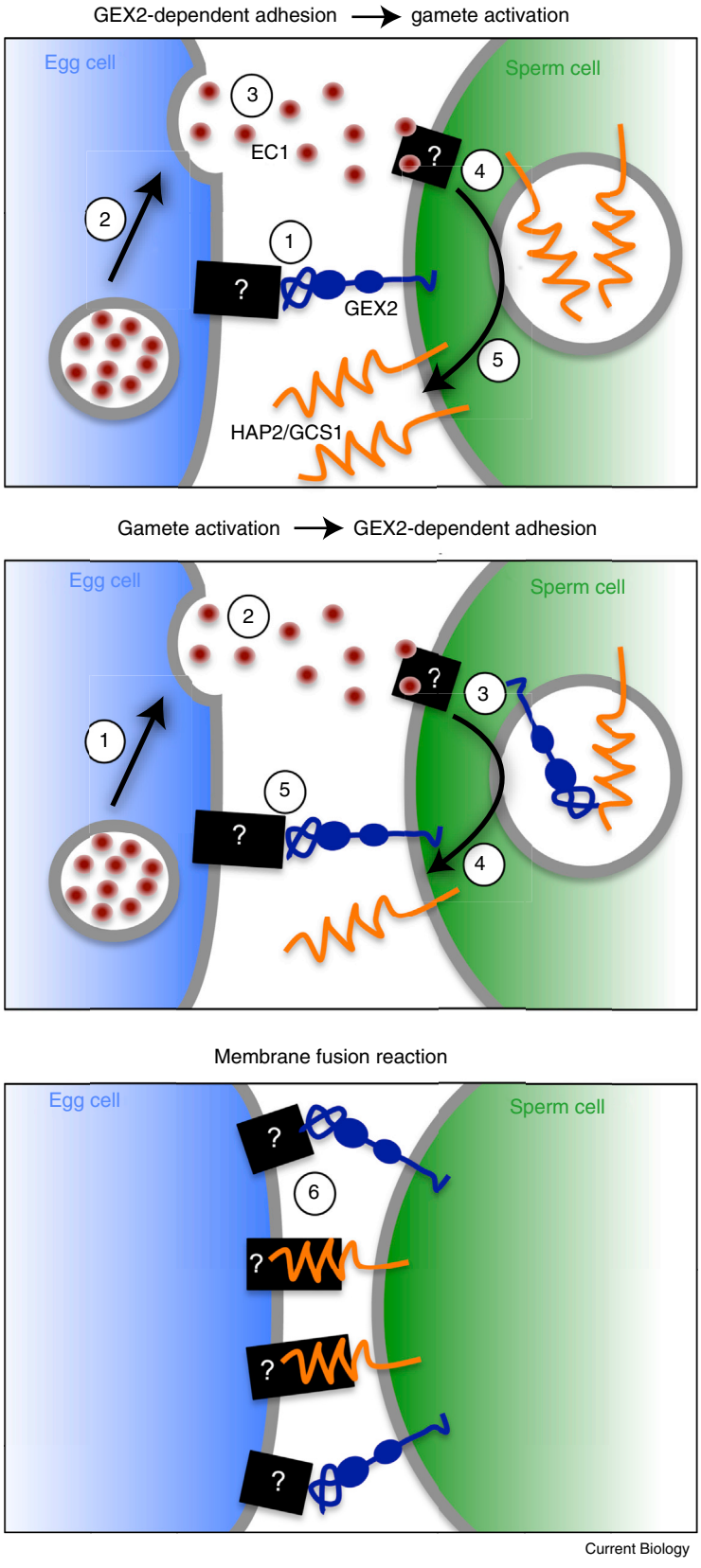

Figure 1. Possible schemes and order of events during gamete interactions in the model plant *Arabidopsis*.

(Top) GEX2 could participate in the initial adhesion (1) that triggers female gamete activation (2) and EC1 release (3) inducing sperm activation (4) leading to exposure of GCS1/HAP1 on the sperm surface. (Middle) GEX2 might alternatively be accessible on the cell surface only after gamete activation and function (1–4) in post-activation adhesion (5) as part of (Bottom) the membrane fusion reaction (6). Numbers indicate order of events and black boxes represent unknown receptors and cell surface interactors.

cytoplasmic vesicles (Figure 1). A report last year resolved this seeming paradox and also uncovered a unifying theme in fertilization — Sprunck and colleagues showed that sperm arrival in the vicinity of both female gametes triggered the egg cell to secrete a set of EC1 proteins, which in turn activated the sperm to expose their previously cryptic HAP2/GCS1 at the cell surface [14]. Thus, as in metazoans and protists, initial interactions between gametes are required to render the gametes competent for cell–cell fusion. In another demonstration of the likely conservation of fertilization mechanisms, members of the tetraspanin family (TET), which are characterized by four transmembrane domains and two extracellular loops, might also be implicated in gamete interaction in plants. TET11 and TET12 localize to the sperm cell plasma membrane and TET9 was detected recently in the plasma membrane of both the egg and central cell [14,15], raising the possibility that they may function as facilitators of the membrane fusion reaction similar to the proposed function of mammalian CD9. The 17 members of the *Arabidopsis* tetraspanin family, however, are expressed throughout the organism and gene disruption studies have not been reported yet.

In this new report, Mori and colleagues [11] present yet another major advance for the field. In a screen for plants defective for double fertilization, they uncovered a male sterile mutant gene whose disruption interfered with fertilization. Several hours after pollination of wild-type ovules with wild-type pollen, the egg and central cell become fertilized by the sperm pairs, and no free sperm pairs are detectable. In the new mutant, however, sperm cell pairs remained visible between the egg and central cell well after pollination. Genetic mapping and mutant rescue experiments showed that the mutation was in *GAMETE EXPRESSED 2* (*GEX2*; At5g49150),

previously identified as an *Arabidopsis* sperm-specific homologue of a maize sperm gene of unknown function [16]. The wild-type *GEX2* gene encodes a single-pass transmembrane protein of 1087 amino acid residues containing two filamin repeat domains and a carboxy-terminal transmembrane domain. Thus, it is likely that almost the entire protein is exposed on the surface of (activated) sperm cells. Importantly, GEX2−GFP rescues fertilization in the mutant. GFP imaging studies showed membrane localization, with some associated with the plasma membrane and substantial amounts internally surrounding the nucleus.

Using a novel cell adhesion assay in which cell wall material was digested *in situ* to generate round gamete protoplasts and in which markers were used to visualize cell boundaries, Mori and colleagues [11] then showed that in some cases one, and in some cases both, *gex2* mutant sperm failed to attach to the egg and central cell protoplasts. This result was in striking contrast to the fusion-defective *hap2/gcs1* mutant, in which both sperm bound to female gamete protoplasts. Thus, the authors concluded that HAP2/GCS1 is required for a step after adhesion and that GEX2 functions in sperm−egg and sperm−central cell adhesion. As the authors indicate, the finding that disruption of *gex2* does not completely block gamete adhesion and fusion indicates that the protein likely acts redundantly with other gene products.

Moreover, the authors' bioinformatic analyses uncovered intriguing properties of GEX2. The ratio between the number of non-synonymous substitutions per non-synonymous site and the number of synonymous substitutions per synonymous site (Ka/Ks), which is a measure of the rate of evolution, showed that GEX2 was diverging more rapidly than HAP2/GCS1. Thus, as in protists, this higher plant gamete adhesion protein likely confers species specificity to sperm−egg/central cell interactions. The most rapidly evolving region of GEX2 proteins from different *Arabidopsis* species turned out to be in the ectodomain in the first filamin repeat domain, which is predicted to form immunoglobulin-like folds found in many cell adhesion proteins [17]. In another nod to conserved features of fertilization, mammalian IZUMO1 also possesses an immunoglobulin-like domain in its predicted ectodomain [5], and the FUS1 protein in *Chamydomonas* also contains an amino-terminal, filamin-like domain in its predicted ectodomain [9]. FUS1 is the species-specific protein expressed only on *plus* gametes of *Chlamydomonas* that is essential for the membrane adhesion step in the *Chlamydomonas* gamete membrane fusion reaction.

These exciting findings have generated a number of new questions. Is GEX2 a *bona fide* adhesion molecule that directly binds to egg cell surface proteins, or does it facilitate the adhesion activity of other as yet unidentified proteins? Which additional proteins are responsible for the residual gamete adhesion observed in the *gex2* mutants? At which step in gamete interactions does GEX2 act — does it act in the initial interactions that trigger egg activation, or does GEX2 act later and become exposed on the sperm surface after gamete activation similar to GCS1/HAP2 (Figure 1)? It can also not be excluded that GEX2 plays a late role in the membrane fusion reaction. It should be straightforward to test whether EC1 is released upon contact with *gex2* mutant sperm and if HAP2/GCS1 is sequestered or located on the surface of *gex2* sperm. The ability to isolate living sperm and female gametes either by FACS or manually [18,19] also now makes *in vitro* experiments possible in *Arabidopsis* to investigate the specificity and strength of the interaction. Finally, the major task for the future is the identification of the GEX2 interaction partner(s) at the egg and central cell surfaces.

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