Arabidopsis thaliana GEX1 has dual functions in gametophyte development and early embryogenesis

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SUMMARY

GEX1 is a plasma membrane protein that is conserved among plant species, and has previously been shown to be expressed in sperm cells and some sporophytic tissues. Here we show that GEX1 is also expressed in the embryo sac before cellularization, in the egg cell after cellularization, in the zygote/embryo immediately after fertilization and in the pollen vegetative cell. We functionally characterize GEX1 in Arabidopsis thaliana, and show that it is a versatile protein that performs functions during male and female gametophyte development, and during early embryogenesis. gex1-1/+ plants, which synthesize a truncated GEX1 mRNA encoding a protein lacking the predicted cytoplasmic domain, but still targeted to the plasma membrane, had embryos that arrested before the pre-globular stage. gex1-3/+ plants, carrying a null GEX1 allele, had defects during male and female gametophyte development, and during early embryogenesis. Using an antisense GEX1 transgenic line we demonstrate that the predicted GEX1 extracellular domain is sufficient and necessary for GEX1 function during the development of both gametophytes. The predicted cytoplasmic domain is necessary for correct early embryogenesis and mediates homodimer formation at the plasma membrane. We propose that dimerization of GEX1 in the zygote might be an upstream step in a signaling cascade regulating early embryogenesis.

Keywords: antisense, embryo sac, pollen, embryo lethal, plasma membrane protein, dimerization, BiFC.

INTRODUCTION

In flowering plants the mature male gametophyte (pollen grain) contains three cells: a large vegetative cell that forms the pollen tube and two sperm cells enclosed within the vegetative cell cytoplasm (McCormick, 2004). The female gametophyte (embryo sac) develops within the ovule and consists of seven cells of four types: three antipodal cells, two synergid cells, one egg cell and one central cell (Sundaresan and Alandete-Saez, 2010). The fertilization process begins when the pollen grain germinates on the female stigmatic cells, and ends when the two sperm cells fertilize the egg and the central cell to form the zygote and the primary endosperm cell, respectively.

Pollen tubes are guided through female tissues (Panalysis et al., 2003) until they turn towards an available ovule. The two synergid cells within the female gametophyte play important roles during pollen tube guidance, as shown in Torenia fournieri (Higashiyama et al., 2001), Zea mays (Okuda et al., 2009), and the Arabidopsis mma1 and maa3 mutants (Shimizu and Okada, 2000). The gametic cells also play a role in pollen tube guidance (von Besser et al., 2006; Chen et al., 2007; Alandete-Saez et al., 2008). The pollen tube enters the micropyle through the filiform apparatus, then releases the two sperm cells within the degenerating synergid cytoplasm (Weterings and Russell, 2004). Pollen tube arrest after entering the micropyle was perturbed by mutations in several genes, including FERONIA (Escobar-Restrepo et al., 2007), ANXUR1/2 (Boisson-Dernier et al., 2009), LORELEI (Capron et al., 2008) and AMC (Boisson-Dernier et al., 2008). In most cases, the two sperm cells will complete double fertilization, although single fertilization events may occur after the discharge of two sperm cells (Ron et al., 2010) or after the discharge of only one sperm-like cell (Nowack et al., 2006; Chen et al., 2008; Frank and Johnson, 2009; Aw et al., 2010). Upon double fertilization the development of the embryo and the endosperm is initiated.
Embryogenesis is a critical step in the life cycle of seed plants (Dumas and Rogowsky, 2008). The zygote undergoes its first unequal cell division to yield an apical cell, which will develop into the embryo proper, and a basal cell, which will form the suspensor (Guitton and Berger, 2005). The complex process of seed development can be conceptually divided into three phases: the first establishes the pattern of the embryo by rapid cell division; the second is characterized by cell expansion and accumulation of reserves; whereas during the third phase, the seed desiccates (Devic, 2008).

Here we show that Arabidopsis GEX1 is a versatile protein that functions during the development of the male and female gametophytes, and during early embryogenesis. GEX1 is expressed in the embryo sac before cellularization, in the egg cell after cellularization, in the zygote/embryo immediately after fertilization and in the male gametophyte, in both the vegetative cell and in the sperm cells. We characterized two different GEX1 mutant alleles: gex1-1 and gex1-3. gex1-1 plants had arrested embryos, whereas gex1-3 plants had defects during male and female gametophyte development, and during early embryogenesis. We demonstrate that the predicted GEX1 extracellular domain is sufficient and necessary for GEX1 function during the development of male and female gametophytes. Finally, using bimolecular fluorescence complementation (BiFC), we show that GEX1 forms homodimers at the plasma membrane through its cytoplasmic domain, and that this cytoplasmic domain is necessary for early embryogenesis.

RESULTS

GEX1 in Arabidopsis (At5g55490) is the ortholog of a maize protein represented by one expressed sequence tag (EST) among 5000 ESTs from a sperm cell cDNA library (Engel et al., 2003, 2005). GEX1 has a predicted topology of a signal peptide, a large extracellular domain (ECD) containing two coiled-coil domains, three transmembrane domains (TMDs) and a short cytoplasmic domain (CD), as shown in Figure 1(a). Consistent with this prediction, a GEX1-eGFP fusion protein was localized at the plasma membrane upon transient expression in onion epidermal cells (Engel et al., 2005), and expression of GEX1 was detected in sperm cells using a ProGEX1:eGFP reporter line (Engel et al., 2005). The sequence of GEX1 is conserved among land plants, including monocots, eudicots, the moss Physcomitrella and the lycophyte Selaginella (Figure S1). Plasma membrane proteins that are expressed in gametic cells might play important roles in recognition and signaling during gametophyte development and/or during different steps of the fertilization process. To determine the role of GEX1 during these stages we used a reverse genetic approach, and characterized two T-DNA insertion lines: gex1-1 and gex1-3. The T-DNA insertions were mapped to two different locations within the open reading frame (ORF) of GEX1 (Figure 1a), potentially disrupting different protein domains.

Characterization of mutant phenotypes

To investigate if GEX1 had a role during the fertilization process, we first determined if the mutant lines had defects in seed development or seed set. We examined siliques of gex1-1/+ and gex1-3/+ plants 6–8 days after pollination (DAP). Both lines showed fewer wild type-like developed seeds than expected. Superficially, the gex-1 and gex1-3 phenotypes looked similar, but there were differences. The gex1-1/+ plants showed approximately 25% reduced seed set (22 ± 5% aborted seeds and 3 ± 1% undeveloped ovules; Figure 1c), whereas the gex1-3/+ plants showed 49% reduced seed set (12 ± 2% aborted seeds and 37 ± 4% undeveloped ovules; Figure 1d). We then examined pollen in open flowers. The gex1-1/+ plants had 100% mature tricellular pollen grains (n = 1399) (Figure S2a,f), but gex1-3/+ plants had 36% aborted pollen (n = 1639) (Figure S2b,g). In order to ascertain the genetic segregation of each mutant allele in self-pollinated progeny, we scored the ratio (resistant versus sensitive) of gex1-1/+ and gex1-3/+ plants, using the linked basta or kanamycin (kan) resistance genes, respectively. For the gex1-1 allele the segregation ratio was 1.82:1 (1135:622), whereas for the gex1-3 allele the ratio was 1.65:1 (896:620).

These data suggested that each mutant allele partially affected the transmission of one or both gametophytes, and/or that the homozygote was embryo lethal. To distinguish among these hypotheses, we carried out reciprocal crosses of gex1-1/+ and gex1-3/+ plants with wild-type (WT) plants, and scored the transmission efficiency of each mutation, by selection on basta or kan, respectively. Table 1 shows that for gex1-1 the transmission of the T-DNA insertion through the female gametophyte was complete (approximately 97%), but that transmission through the male gametophyte was slightly reduced (approximately 78%). When pollen from these plants was crossed with WT females, the resulting siliques (6–8 DAP) showed 41% transmission of the mutant allele through the female gametophyte. Both lines showed fewer wild type-like developed seeds than expected. Superficially, the gex1-1/+ and gex1-3/+ phenotypes looked similar, but there were differences. The gex1-1/+ plants showed approximately 25% reduced seed set (22 ± 5% aborted seeds and 3 ± 1% undeveloped ovules; Figure 1c), whereas the gex1-3/+ plants showed 49% reduced seed set (12 ± 2% aborted seeds and 37 ± 4% undeveloped ovules; Figure 1d). We then examined pollen in open flowers. The gex1-1/+ plants had 100% mature tricellular pollen grains (n = 1399) (Figure S2a,f), but gex1-3/+ plants had 36% aborted pollen (n = 1639) (Figure S2b,g). In order to ascertain the genetic segregation of each mutant allele in self-pollinated progeny, we scored the ratio (resistant versus sensitive) of gex1-1/+ and gex1-3/+ plants, using the linked basta or kanamycin (kan) resistance genes, respectively. For the gex1-1 allele the segregation ratio was 1.82:1 (1135:622), whereas for the gex1-3 allele the ratio was 1.65:1 (896:620).

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When gex1-3/+ plants were used as females, however, the siliques had 62 ± 8% WT seeds, 36 ± 7% undeveloped ovules and 1 ± 1% aborted seeds (n = 211): this data is consistent with the reduced (41%) transmission of the gex1-3 allele through the female gametophyte.

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To determine if all embryo sacs were targeted by pollen tubes, we used decolorized aniline blue (DAB) to visualize pollen tubes in the pistils of gex1-1/+ and gex1-3/+ plants. We collected self-pollinated flowers at 1–2 DAP and stained the pistils with DAB. In gex1-1/+ plants all ovules were reached by pollen tubes (Figure 1e), which is consistent with the full transmission of the gex1-1 allele through the female. In the gex1-3/+ plants 35/6% (n = 615) of the ovules did not attract pollen tubes (Figure 1f–h), consistent with the reduced transmission of the gex1-3 allele through the female. To confirm the female gametophyte defect we crossed pollen of the ProLAT52:GUS (Twelli et al., 1991) reporter line as a male. A fertilized ovule (black arrow points to a blue pollen tube at the micropyle), and a putative gex1-3 ovule (red arrow) that failed to attract a pollen tube towards the micropyle.

Table 1 Transmission Efficiency in gex1-1/+ and gex1-3/+ plants.

<table>
<thead>
<tr>
<th>Parental Genotype (Female X Male)</th>
<th>BastaR</th>
<th>BastaS</th>
<th>Transmission Efficiency (TE) (%)</th>
<th>c2 Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>gex1-1/+ X WT</td>
<td>125</td>
<td>129</td>
<td>97</td>
<td>c2 = 0.063, P = 0.8018</td>
</tr>
<tr>
<td>WT X gex1-1/+</td>
<td>165</td>
<td>207</td>
<td>78</td>
<td>c2 = 4.742, P = 0.0294*</td>
</tr>
<tr>
<td>gex1-3/+ X WT</td>
<td>73</td>
<td>175</td>
<td>41</td>
<td>c2 = 41.952, P &lt; 0.0001*</td>
</tr>
<tr>
<td>WT X gex1-3/+</td>
<td>74</td>
<td>156</td>
<td>47</td>
<td>c2 = 29.235, P &lt; 0.0001*</td>
</tr>
</tbody>
</table>

Transmission efficiency = Resistant/ Sensitive × 100.

*TE is statistically significant from the 100% TE expected for a sporophytic mutation.
mature female gametophytes in arrested at the FG1 stage (Figure 2c,e,d, respectively). All eight nuclei) stage embryo sacs, and embryo sacs that had WT-like FG5 (eight nuclei) and FG6 (seven-celled and FG7) in emasculated pistils of (a) Mature wild-type embryo sac after cellularization and differentiation (stage FG7) in emasculated pistils of gex1-3/+ plants. (b) Arrested embryo sac at FG1 in an ovule from the same pistil as in (a). (c) Wild-type embryo sac at FG5 in pistils of gex1-3/+ plants. (d) Arrested embryo sac at FG1 in an ovule from same pistil as (c). (e) Wild-type embryo sac at FG6 in pistils of gex1-3/+ plants. (f) Mature wild-type embryo sac after cellularization (stage FG7) in emasculated pistils of gex1-1/+ plants. Ccn, central cell nucleus; Ecn, egg cell nucleus; FM, functional megaspore; Syn, synergid nucleus. Nuclei are false-colored.

Figure 2. Female gametophyte development in gex1-1/+ and gex1-3/+ plants. (a) Mature wild-type embryo sac after cellularization and differentiation (stage FG7) in emasculated pistils of gex1-3/+ plants. (b) Arrested embryo sac at FG1 in an ovule from the same pistil as in (a). (c) Wild-type embryo sac at FG5 in pistils of gex1-3/+ plants. (d) Arrested embryo sac at FG1 in an ovule from same pistil as (c). (e) Wild-type embryo sac at FG6 in pistils of gex1-3/+ plants. (f) Mature wild-type embryo sac after cellularization (stage FG7) in emasculated pistils of gex1-1/+ plants. Ccn, central cell nucleus; Ecn, egg cell nucleus; FM, functional megaspore; Syn, synergid nucleus. Nuclei are false-colored.

reporter line with gex1-3/+ plants: 37 ± 9% ovules (n = 209) were not reached by blue pollen tubes (Figure 1i,j) in siliques at 1–2 DAP. Because defects in female gametophyte development can cause a failure in pollen tube attraction (Marton and Dresselhaus, 2010), we decided to analyze mature female gametophytes of gex1-3/+ plants to determine if there were abnormalities in cell polarity and/or in the position of the nuclei. Pistils of emasculated gex1-3/+ plants had 63 ± 7% (n = 354) WT-like four-celled embryo sacs (FG7 stage) (Sundaresan and Alandete-Saez, 2010), but 36 ± 7% of the embryo sacs had arrested at the functional megaspore stage (FG1 stage) (Figure 2a,b, respectively). Younger pistils dissected from closed buds of gex1-3/+ plants also carried both WT-like FG5 (eight nuclei) and FG6 (seven-celled and eight nuclei) stage embryo sacs, and embryo sacs that had arrested at the FG1 stage (Figure 2c,e,d, respectively). All mature female gametophytes in gex1-1/+ plants showed correct cell polarity and nuclei position (Figure 2f). These results suggest that putative gex1-3 female gametophytes were arrested at the FG1 stage, a phenotype that explains the reduced ability to attract pollen tubes observed in gex1-3/+ plants.

**gex1-1/gex1-1 and gex1-3/gex1-3 affect early embryo development**

The transmission and seed set results indicated that gex1-1 and gex1-3 mutant phenotypes were not fully penetrant, and therefore both the male and female gametophytes could transmit the mutant allele to the next generation. It should therefore be possible to obtain plants homozygous for these alleles. We selected seeds from self-pollinated gex1-1/+ and gex1-3/+ plants on basta or kan, respectively, and genotyped over 100 seedlings from each line. No homozygotes were identified for either mutant, suggesting that gex1-1/gex1-1 and gex1-3/gex1-3 are embryo lethal, which was consistent with the aborted seeds found in siliques of both mutant lines. To determine the stage of embryogenesis that was compromised in the putative gex1-1/gex1-1 and gex1-3/gex1-3 embryos, we dissected and cleared seeds from siliques at different developmental stages and visualized them using differential interference contrast (DIC) imaging. Figure 3(a,b) shows normal embryo development at the triangular stage (5 DAP), and Figure 3(c,d) shows normal embryo development at the torpedo stage (6–7 DAP). Figure 3(e,f) shows abnormal embryo development in a putative gex1-1/gex1-1 seed from the same silique as Figure 3(a), whereas Figure 3(g) and 3(h) show seeds with abnormal embryo development from the same silique as Figure 3(c). After the analysis of over 100 seeds showing abnormal embryo development, we observed that these embryos arrested between the two- and eight-celled embryo stage, and showed altered cell size in most cases, although abnormal arrested embryos at later developmental stages were also found. These seeds were slightly smaller than other seeds in the same silique, and showed endosperm nuclear divisions. We saw a similar arrested embryo development phenotype in a few seeds of self-pollinated gex1-3/+ plants at 5 DAP (Figure 3i,j). To confirm that the endosperm development observed in the aborted seeds resulted from a product of fertilization, and not from autonomous endosperm development, we introgressed the reporter gene line ProFAC1:GUS, which is expressed in both embryo and endosperm upon fertilization (Xu et al., 2005), into the gex1-1/+ background. Figure 3(k) shows a WT developing seed that has GUS signal in the embryo and endosperm at 2 DAP. Figure 3(L) shows a putative gex1-1/gex1-1 seed with GUS expression in the arrested embryo and developing endosperm at 2 DAP. We conclude that the putative gex1-1/gex1-1 zygote/embryo arrested development soon after double fertilization, even though the endosperm continued developing (for at least 6–7 DAP) before the seed finally aborted.

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We demonstrated that the phenotypes observed in the two mutant lines were caused by the corresponding T-DNA insertions by introducing a construct carrying the GEX1 WT allele driven by its own promoter into the two mutant backgrounds. In each case, the phenotypes were fully complemented (Figure S3).

**GEX1 is expressed in male and female gametophytes**

The phenotypes identified in the insertion lines suggested a role for GEX1 in the female and male gametophyte, and in the embryo. To further evaluate GEX1 expression, we generated the GUS reporter construct ProGEX1:GUS. A GUS signal was detected in the embryo sac before cellularization (a very weak signal at the FG1/FG2 stage and a more visible signal at the FG3/FG4/FG5 stage), in egg cells of mature female gametophytes and in zygotes/embryos of ovules at 1–2 DAP, but not in embryos older than 2 DAP (Figure 4a–f). This data is consistent with the gene expression map of Arabidopsis embryo development (http://www2.bri.nrc.ca/plantembryo) (Xiang *et al.*, 2011), which reported high expression levels of GEX1 in the elongated zygote through the octant embryo stage. We also detected GUS in the vegetative cell, in a few pollen tubes upon pollen germination, and in root tips (Figure S4). RT-PCR using RNA from different tissues (Figure 4g) showed that GEX1 was expressed strongly in pollen and weakly in unpollinated pistils. These additional expression data support the role of GEX1 during gametophyte development and early embryogenesis inferred from the gex1-1 and gex1-3 mutant lines.
We performed quantitative real-time PCR (qRT-PCR) to analyze GEX1 expression levels in the WT and in the mutant lines. We first extracted RNA from pollen and unpollinated pistils of gex1-1/+ plants, and observed that GEX1 mRNA levels were approximately 50% of WT levels in both (Figure 4h). Because the level of GEX1 expression could be detected equally in pollen and unpollinated pistils, we used RNA from unpollinated pistils for further analysis, as they were easier to collect. The mRNA levels of GEX1 in unpollinated pistils of gex1-3/+ plants were approximately 50% of the levels found in WT plants (Figure 4h). In addition, we quantified GEX1 expression with a third allele, gex1-2. The expression of GEX1 in unpollinated pistils, anthers and leaves, and in pistils at 3 DAP, was higher in gex1-2/+ plants than in the WT (Figure S5). The T-DNA insertion in gex1-2/+ plants (FLAG line) contained a CaMV35S promoter immediately before the left border, and we therefore reasoned that higher expression of GEX1 could be caused by such a promoter, as has happened for other T-DNA insertion lines (Williams et al., 2005). We did not characterize gex1-2 further.

**gex1-1/+ plants express a truncated GEX1 protein** (gex1-1) that localizes to the plasma membrane

We thought that the differences observed between the two alleles might be related to the position of the T-DNA insertion within the open reading frame (ORF) of GEX1, and we hypothesized that gex1-1/+ plants might synthesize a truncated GEX1 mRNA, up to the T-DNA insertion (Figure 5a), that might be translated and localized at the plasma membrane. To test this hypothesis we first determined GEX1 mRNA levels using primers located upstream of the T-DNA insertion in gex1-1/+ plants. qRT-PCR analysis showed that GEX1 mRNA levels in unpollinated pistils were similar to WT levels (Figure 5b), suggesting that a partial GEX1 mRNA was probably transcribed up to the location of the T-DNA insertion; when we used primers located downstream of the insertion we observed that GEX1 mRNA expression was decreased to 50% of WT levels (Figures 4h and 5b). In addition, 3' rapid amplification of cDNA ends (3'-RACE) using RNA from unpollinated pistils of gex1-1/+ plants detected two bands (Figure 5c), one corresponding to full-length GEX1 mRNA and the second to a truncated GEX1 mRNA up to the T-DNA insertion location, and including 51 nucleotides carrying a sequence that is complementary to the left border of the T-DNA, which would encode 16 amino acids before a stop codon.

Because this GEX1-1 truncated mRNA was polyadenylated, we tested if the partial mRNA synthesized in gex1-1/+ plants could be translated into a truncated protein lacking 129 amino acids (illustrated in Figure 5a), and if so, to determine its subcellular localization. We bombarded onion cells with a construct encoding the putative truncated version fused to GFP at the C terminus and under the control of the CaMV35S promoter (Pro35S:gex1-1-eGFP). Figure 5(d) shows that the WT version of GEX1 is located at the plasma membrane, as has already been shown by Engel et al. (2005). We also detected some perinuclear signal, most likely corresponding to the endoplasmic reticulum (Beraud-Dufour and Balch, 2002), and probably to the result of excess fusion protein in the exocytic pathway (Roselli et al., 2004). Figure 5(f) shows that the truncated mRNA was translated into a protein that was also targeted to the plasma
membrane. The gex1-1-eGFP fusion protein also showed a weak perinuclear signal.

The truncated gex1-1 protein is equivalent to GEX1 during gametophyte development but not during early embryogenesis

Because gex1-1/+ plants did not show pollen tube guidance defects or embryo sac arrest, nor the pollen abortion observed in gex1-3/+ plants, we hypothesized that the truncated gex1-1 protein (Figure 5a) might be equivalent to GEX1 during male and female gametophyte development before double fertilization. If so, silencing the expression of the gex1-1 truncated mRNA in the male and female gametophytes would phenocopy the defects observed in gex1-3/+ plants. To test this hypothesis we generated transgenic lines silencing GEX1 expression only in the gametophytes by using an antisense construct driven by the GEX2 promoter that is not active in sporophytic tissues (Engel et al., 2005), nor in the zygote or embryo, according to the gene expression map of Arabidopsis embryo development (http://www2.bri.nrc.ca/plant-embryo; Xiang et al., 2011), but is expressed both in the sperm (Engel et al., 2005) and in the egg (Alandete-Saez et al., 2008).

We identified 15 primary (T1) transformants that showed differing degrees of reduced seed set, i.e. approximately 25–65% undeveloped ovules. Because plants showing more than 50% reduced seed set might carry multiple T-DNA insertions, we grew T2 and T3 segregating plants from four different AS families (AS5, AS55, AS11 and AS35) that had shown up to 50% reduced seed set (Figure S6), and then quantified GEX1 mRNA levels in unpollinated pistils and stamens using qRT-PCR. A silique of one such antisense plant (AS5) at 6–8 DAP, with 50% undeveloped ovules (Table 2), is shown in Figure 6(b). Figure 6(c,d) shows that GEX1 expression was reduced to approximately 50% in all four lines in both tissues. We collected self-pollinated pistils from these four lines at 1–2 DAP and stained them with DAB. In all four lines undeveloped ovules did not attract pollen tubes, a phenotype similar to that observed in gex1-3/+ plants (Figure 6e).

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Table 2 Seed set in mature siliques (6–8 DAP) of Col, gex1-1/+, AS5/- and gex1-1/+; AS5/-.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Developed (%)</th>
<th>Aborted (%)</th>
<th>Undev. (%)</th>
<th>Total (n)</th>
<th>c2 Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>1026 (98)</td>
<td>20 (2)</td>
<td>0</td>
<td>1046</td>
<td>N/A</td>
</tr>
<tr>
<td>gex1-1/+</td>
<td>769 (75)</td>
<td>225 (22)</td>
<td>31 (3)</td>
<td>1025</td>
<td>c2 = 2840.2, P &lt; 0.0001*</td>
</tr>
<tr>
<td>AS5/-</td>
<td>929 (49)</td>
<td>19 (1)</td>
<td>947 (50)</td>
<td>1895</td>
<td>c2 = 23126.4, P &lt; 0.0001*</td>
</tr>
<tr>
<td>gex1-1/+;AS5/-</td>
<td>470 (49)</td>
<td>109 (10)</td>
<td>514 (47)</td>
<td>1094</td>
<td>c2 = 23126.4, P &lt; 0.0001*</td>
</tr>
</tbody>
</table>

*Numbers of aborted and undeveloped seeds are statistically significant from that in Col plants.
that WT plants had full seed set, gex1-1/+ plants had 22 ± 5% aborted seeds, GEX1 AS/– plants had 50 ± 5% undeveloped ovules and GEX1 AS/–;gex1-1/+ plants had 47 ± 6% undeveloped ovules and 10 ± 2% aborted seeds. These aborted seeds were putative gex1-1/gex1-1 seeds, either from the fertilization of embryo sacs (gex1-1) with sperm cells carrying the gex1-1 allele, but lacking the GEX1 AS construct (gex1-1/–), or from the small proportion of pollen grains carrying both the gex1-1 allele and the AS construct (gex1-1/–;GEX1 AS) that did not abort and were able to reach gex1-1 embryo sacs. Undeveloped ovules in the self-pollinated pistils of GEX1 AS/–;gex1-1/+ plants at 1–2 DAP failed to attract pollen tubes, as did the GEX1 AS5 line, and had 36% pollen abortion (Figure S2d; Table S1). We did not recover a gex1-1/gex1-3 plant from crosses using gex1-1/– as a female and gex1-1/– as a male.

These results indicated that silencing GEX1 expression using an antisense construct phenocopied the gex1-3 mutation, and also that the truncated gex1-1 protein expressed in gex1-1/+ plants was equivalent to the WT GEX1 protein during the development of male and female gametophytes, even though the gex1-1 truncated protein lacks the 129 amino acid cytoplasmic domain. However, putative gex1-1/– gex1-1/– embryos were arrested at an early stage because of the absence of the GEX1 cytoplasmic domain.

GEX1 forms homodimers interacting through the cytoplasmic domain

Because it is common that proteins located at membranes form dimers to carry out their functions, we hypothesized that GEX1 might form homodimers in the plasma membrane. To test this hypothesis, we used the BiFC assay (Citovsky et al., 2006) and transient expression in onion epidermal cells. The full length (FL) amino acid sequence of GEX1 was fused to either the N terminus or C terminus of yellow fluorescent protein (YFP) as a C-terminal fusion. Figure 7(a) shows that WT GEX1 (FL) interacts with itself in the plasma membrane, indicating that GEX1 forms homodimers when expressed in the same cell. We next tested if the truncated gex1-1 protein could dimerize with either the WT GEX1 or another gex1-1 protein (Figure 7b,c). In neither case was a fluorescent signal detected in the plasma membrane, even though the bombardment assay worked efficiently (many transformed cells expressed the Pro3S:S:GUS construct that was co-bombarded with the spYFP vector). That the gex1-1 protein could not interact with GEX1 or another gex1-1 protein suggested that the interaction to form homodimers occurred through the transmembrane domains, through the cytoplasmic domains or through both. We therefore made a new construct encoding the signal peptide at the N terminus, followed by the three transmembrane domains and the cytoplasmic domain, fused to either N-YFP or C-YFP. Figure 7(d,e) shows that this protein could interact with both full-length WT GEX1 and with itself, confirming that GEX1
forms homodimers through its transmembrane and cytoplasmic domains. We observed perinuclear fluorescence with all GEX1 protein versions that showed positive interactions in the BiFC assay, which was consistent with the subcellular localization of both the full-length GEX1 and gex1-1 truncated proteins. In addition, we tested whether the extracellular domain (ECD) of GEX1 could interact with itself in the opposite orientation, because that would suggest that GEX1 could form homodimers when in the plasma membrane of two different cells (e.g., egg and sperm cells, before or during the fusion of their membranes). We used the predicted ECD of GEX1, without the predicted signal peptide, fused to either the N terminus or the C terminus of YFP as C-terminal or N-terminal fusions, but did not detect any interaction between the extracellular domains.

**DISCUSSION**

GEX1 was previously shown to be expressed in sperm cells (Engel et al., 2005; Borges et al., 2008; Slotkin et al., 2009) as well as in other sporophytic tissues using a GFP reporter construct (Engel et al., 2005). Here we used a more sensitive GUS reporter to show that GEX1 is also expressed during early embryo sac development and in the egg cell, but also during the first divisions of the zygote/embryo (Figure 4a–d). We did not detect GUS signal in the sperm cells, possibly because expression in the vegetative cell masked the sperm cell signal, or because the promoter used for the GUS reporter construct (same promoter used in the complementation constructs) was slightly smaller than that used for the ProGEX1-eGFP construct (upstream of the ATG at 1606 versus 1808 bp). Using a reverse genetic approach we showed that GEX1 is a versatile protein of dual function during gametophyte development and early embryogenesis. Our genetic approach was unable to uncover a role for GEX1 in sperm cells during fertilization, because of its earlier role during pollen development, although that possibility cannot be ruled out. The gex1-3 T-DNA insertion, located in the fourth exon (Figure 1a), disrupts GEX1 mRNA transcription before the sequence encoding its transmembrane domain and, based on qRT-PCR data, is a null allele. gex1-3/+ plants showed defects in pollen (Figure S2), embryo sac (Figure 2) and early embryo development (Figure 3). Although it is well documented that the embryo transcriptome shares a high number of expressed genes with both...
the male and female gametophytes (Haecker et al., 2004; Berg et al., 2005; Jenik et al., 2007; Spencer et al., 2007; Bayer et al., 2009; Wuest et al., 2010), there is limited information linking the expression pattern of a gametophytically expressed gene with a role during early embryogenesis (Bayer et al., 2009). The embryo sacs carrying the gex1-3 allele showed arrested development at the FG1 stage (Figure 2), and did not attract pollen tubes (Figure 1f); most likely because of the absence of attractant molecules required for microspor pollen tube guidance, which are only secreted when female gametophytes are fully mature (Higashiyama, 2002; Palanivelu and Preuss, 2006; Dresselhaus and Marton, 2009). In contrast to mutations in other sperm-expressed genes (von Besser et al., 2006; Mori et al., 2006; Nowack et al., 2006; Chen et al., 2008; Ron et al., 2010), gex1-3 pollen grains did not show defects in pollen tube guidance, pollen tube reception or fertilization. Instead, gex1-3 pollen grains aborted, suggesting that GEX1 is essential for correct pollen maturation, as is the case for other pollen-expressed genes (Chen and McCormick, 1996; Boavida et al., 2007; Bayer et al., 2009).

Half of the gametophytes produced by gex1-1/+ plants expressed the WT full-length GEX1 mRNA, and the other half expressed truncated gex1-1 mRNA. We showed that both transcripts (GEX1 and gex1-1) could be translated into proteins that were targeted to the plasma membrane (Figure 5c). gex1-1/+ plants did not have the gametophytic defects observed in gex1-3/+ plants. Three lines of evidence support the hypothesis that the predicted extracellular domain of GEX1, when inserted at the plasma membrane, is sufficient and necessary for the development of both male and female gametophytes: (i) gex1-1/+ plants had aborted seeds because of embryo arrest, and not the gametophytic defects observed in gex1-3/+ plants (Figure 3); (ii) transgenic lines carrying a GEX1 antisense construct phenocopied the gametophytic defects observed in gex1-3 plants, consistent with the downregulation of GEX1 transcripts (Figure 6); and (iii) the introduction of the antisense construct in the gex1-1 background phenocopied the gametophytic defects caused by the gex1-3 mutation, which is consistent with the downregulation of GEX1 and gex1-1 transcripts (Table 2). GEX1 formed homodimers in the plasma membrane, and its predicted cytoplasmic domain (CD) was sufficient and necessary for GEX1 interaction and function during early embryogenesis (Figure 7). However, the fully functional gametophytes found in gex1-1/+ plants indicate that the role of the predicted extracellular domain (ECD) of GEX1 during gametophyte development does not require GEX1 dimerization. Although we did not obtain evidence that the extracellular domain of GEX1 can interact with itself when in opposite orientations, these BiFC experiments cannot rule out the possibility that GEX1 can form homodimers when in the plasma membrane of two different cells, because the interaction might require specific protein folding in the plasma membrane. Coiled-coil domains are found in diverse proteins and mediate protein–protein interactions (Suzuki et al., 2008). The presence of two coiled-coil domains in the predicted extracellular domain of GEX1 (Figure 1a) support the idea that the extracellular domain might interact with itself or with other proteins.

The strong GUS signal observed in embryos at 1–2 DAP (Figure 4d) and microarray expression data reported in the gene expression map of Arabidopsis embryo development (http://www2.bri.nrc.ca/plantembryo; Xiang et al., 2011) support the speculation that de novo transcription/translation of GEX1 occurs immediately after karyogamy, instead of by protein diffusion in the plasma membrane after fusion of the gametic cells, as is true for other plasma membrane proteins (Hink et al., 2008). The de novo translated GEX1 would then form dimers in the plasma membrane through its cytoplasmic domain, and we hypothesize that this dimerization would be required for an upstream step in a signaling cascade involved in the establishment and development of the early embryo. It would be interesting to investigate if the homeobox transcription factors WOX2 and WOX8, which act as complementary cell fate regulators in the apical and basal lineages during early embryogenesis (Breuninger et al., 2008), and are co-expressed in both the egg cell and zygote (Haecker et al., 2004), might be downstream players in this signaling cascade.

**EXPERIMENTAL PROCEDURES**

Primers for cloning and genotyping are listed in Table S2.

**Plant materials and growth conditions**

Arabidopsis plants (Columbia, Col-0) were grown in a glasshouse as described by Alandete-Saez et al. (2008). Seeds were plated on MS medium containing 30 μg ml⁻¹ BASTA (gex1-1/+ and GEX1 AS plants) or 50 μg ml⁻¹ kanamycin (gex1-2/+ and gex1-3/+ plants), and the resulting seedlings were scored for resistance 2 weeks later.

**T-DNA line and sequence analyses**

The left borders of the T-DNA insertions CS817262 (gex1-1), FLAG_4BE09 (gex1-2) and FLAG_081B03 (gex1-3) were amplified by PCR and sequenced to verify the insertion sites. The structure and subcellular localization of GEX1 were predicted as described in Alandete-Saez et al. (2008).

**Cloning and generation of transgenic lines**

All constructs were subcloned into pENTR 3C or pENTR/D-TOPO vectors (Invitrogen, http://www.invitrogen.com), confirmed by sequencing and transferred to destination vectors by an LR reaction using LR Clonase II (Invitrogen). The full-length GEX1 open reading
frame was amplified from Arabidopsis pollen cDNA using primers MA7/M8, and was cloned in an antisense orientation into pB7WG2*-ProGEX2, as in (Alandete-Saez et al., 2008). The GEX1 promoter (1.6 kb) was amplified using primers MA9/MA10 and cloned into pBPGWF7 (Karimi et al., 2002) to generate the reporter line ProGEX1:eGFP-GUS. For the complementation construct a 3.8-kb fragment of GEX1, including the promoter and 3’ untranslated region (UTR), was amplified from genomic DNA using primers MA11/MA12 and cloned into a modified pK2GW7 (for gex1-1 complementation) or pH2GW7 (for gex1-3 complementation) vectors (Karimi et al., 2002), from which the CaMV35S promoter had been removed to generate ProGEX1:GEX1 complementation constructs.

Phenotypic analysis and complementation of gex1-1/+ and gex1-3/+ plants
Phenotypic analyses of seed set and reciprocal crosses were performed as described in Alandete-Saez et al. (2008). Decolorized aniline blue (DAB) staining was performed as described in Mori et al. (2006). GUS expression analyses were performed as described in Johnson et al. (2004), and seed clearings were performed as described in Pagnussat et al. (2005). To complement the gex1-1 mutant, the ProGEX1:GEX1 complementation construct was introduced into Col-0 plants using Agrobacterium transformation (Clough and Bent, 1998), then pollen from transgenic T1 lines was used for crosses with gex1-1/+. Seedlings from these crosses were selected on kanamycin and were genotype for the presence of the gex1-1 T-DNA. Five independent ProGEX1:GEX1; gex1-1/− F1 plants were taken to the next generation. Seeds were selected on kanamycin and homozygous gex1-1/−;gex1-1/− GEX1 complementation plants were identified by PCR. To complement the gex1-3 mutant, the ProGEX1:GEX1 complementation construct was introduced into gex1-3/+ plants. T1 lines were selected on hygromycin and geno- typed for the presence of the gex1-3 T-DNA. Positive T1 plants were allowed to self and T2 seedlings were selected on both hygromycin and kanamycin. Homozygous gex1-3/gex1-3/GEX1 complementation plants were identified by PCR.

RT-PCR and real-time PCR
RNA was extracted from different tissues using an RNeasy plant miniprep kit (Qiagen, http://www.qiagen.com) with on-column DNase treatment. cDNA was synthesized using 20-mer oligod(T) and Superscript III Reverse Transcriptase (Invitrogen). RT-PCR was performed using 1 μg of total RNA. Real-time PCR primer pairs were designed using primer express (Applied Biosystems, http://www.appliedbiosystems.com). IPP2 (At3g02780) was used for normalization, as described in Alandete-Saez et al. (2008). Amplification was performed with a MyIQ Real-Time PCR Detection System (Bio-Rad, http://www.bio-rad.com), with 1.5 μg and 500 ng of total RNA from unpollinated pistils and anthers, respectively.

Generation of clones for subcellular localization and BiFC assays
For subcellular localization, the open reading frames of GEX1 or of gex1-1, without the stop codons, were amplified from cDNA prepared from unpollinated pistils and cloned into pB7FWG2 (Karimi et al., 2002) to generate Pro35S:GEX1:eGFP and Pro35S:GEX1-1-eGFP. To generate constructs for BiFC, all fragments were amplified from cDNA prepared from unpollinated pistils and cloned into either pSATA4-nYFP-N1 or pSATA4-cYFP-N1 (Citovsky et al., 2006). The full-length GEX1 sequence was amplified using the primers MA30/MA31 and the truncated gex1-1 was amplified using primers MA30/MA32. The predicted GEX1 ECD without the signal peptide was amplified using primers MA33/MA34 or MA35/MA36. The cytoplasmic domain construct was cloned by overlapping PCR. The signal peptide (36 amino acids) was amplified with the primers MA37/MA38 and the sequence encoding the transmembrane and cytoplasmic domains was amplified using the primers MA39/MA40. The amplicons were gel-purified and 0.5 μl of each fragment was used as template for a second PCR reaction (50 μl) with the primers MA37/MA40 to amplify the full-length overlapping sequence. These constructs were used for particle bombardment in onion epidermal cells. Microcarriers were prepared with 5 μg of plasmid for subcellular localization or 2 μg of each construct (i.e. nYFP-fused and cYFP-fused) for BiFC, including 500 ng of the Pro35S:GUS construct, mixed and coated onto 1.0-mm gold particles (Seahell Technology, http://www.seahelltech.com) according to the manufacturer’s protocol. The particles were bombarded into pemedepidermal layers of onion using a PDS-100/He particle delivery system (Bio-Rad). The epidermal layers were incubated in the dark at 22°C and observed 16–20 h after bombardment using a Zeiss Axiosvert microscope (Zeiss, http://www.zeiss.com). As negative controls, each protein-nYFP and protein-cYFP fusion construct was bombarded with the complementary half-YFP vector; both empty vectors were also tested.

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of this article:
Figure S1. Amino acid sequence alignment of GEX1.
Figure S2. Pollen phenotypes of gex1-1/−; gex1-3/−, GEX1 AS/− and gex1-1/−;GEX1 AS/− plants.
Figure S3. Complementation of gex1-1 and gex1-3 phenotypes.
Figure S4. GEX1 expression in plants carrying the ProGEX1:GUS construct.
Figure S5. Quantification of GEX1 mRNAs levels by qRT-PCR in plants carrying the gex1-1 T-DNA insertion.
Figure S6. Seed formation in mature siliques of four independent GEX1 AS lines.
Table S1. Pollen in open flowers of Col, gex1-1/−, AS5− and gex1-1/−; AS5− plants.
Table S2. Primers used in this study.

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