Green Sperm. Identification of Male Gamete Promoters in Arabidopsis[1][w]

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Previously, in an effort to better understand the male contribution to fertilization, we completed a maize (*Zea mays*) sperm expressed sequence tag project. Here, we used this resource to identify promoters that would direct gene expression in sperm cells. We used reverse transcription-polymerase chain reaction to identify probable sperm-specific transcripts in maize and then identified their best sequence matches in the Arabidopsis (*Arabidopsis thaliana*) genome. We tested five different Arabidopsis promoters for cell specificity, using an enhanced green fluorescent protein reporter gene. In pollen, the *AtGEX1* (*At5g55490*) promoter is active in the sperm cells and not in the progenitor generative cell or in the vegetative cell, but it is also active in ovules, roots, and guard cells. The *AtGEX2* (*At5g49150*) promoter is active only in the sperm cells and in the progenitor generative cell, but not in the vegetative cell or in other tissues. A third promoter, *AtVEX1* (*At5g62580*), was active in the vegetative cell during the later stages of pollen development; the other promoters tested (*At1g66770* and *At1g73350*) did not function in pollen. Comparisons among *GEX1* and *GEX2* homologs from maize, rice (*Oryza sativa*), Arabidopsis, and poplar (*Populus trichocarpa*) revealed a core binding site for Dof transcription factors. The *AtGEX1* and *AtGEX2* promoters will be useful for manipulating gene expression in sperm cells, for localization and functional analyses of sperm proteins, and for imaging of sperm dynamics as they are transported in the pollen tube to the embryo sac.

In an effort to identify proteins potentially involved in fertilization, we sequenced approximately 5,000 expressed sequence tags (ESTs) from maize (*Zea mays*) sperm (Engel et al., 2003). We found that maize sperm cells contain a diverse complement of transcripts. Most sperm transcripts tested were also expressed in other cell types, but we identified several that appeared to be sperm specific in mature pollen. For functional analyses of sperm proteins, promoters that direct gene expression in sperm cells are needed.

A number of pollen-specific promoters have been isolated from plants, but most are active only in the vegetative cell of the pollen grain and not in the sperm cells (e.g. Twell et al., 1990; Huang et al., 1996; Voronin et al., 2001). However, there are a few promoters known that drive reporter gene expression in generative cells and in sperm. For example, the promoter of the *LILY GENERATIVE CELL-SPECIFIC 1* (*LGC1*) gene of lily (*Lilium longiflorum*) directs reporter gene expression in the generative cells and sperm cells of transgenic *Nicotiana tabacum* (Singh et al., 2003). In situ hybridization in lily showed that the *LGC1* mRNA is present in both generative cells and sperm cells (Xu et al., 1999). An Arabidopsis (*Arabidopsis thaliana*) MYB transcription factor gene, *DUO POLLEN 1* (*DUO1*), is specifically expressed in the generative cells and sperm cells, as shown by a promoter fusion to a nuclear-targeted mutated red fluorescent protein (Rotman et al., 2005).

We wanted to identify promoters that would be useful for driving expression of reporter genes in the sperm cytoplasm. For imaging of sperm dynamics, the ideal promoters should not be active in the vegetative cell of the pollen grain. We selected genes in Arabidopsis that were similar to several different sperm-specific transcripts from maize. The putative promoter regions of five Arabidopsis genes were tested, by expressing enhanced green fluorescent protein (eGFP) in transgenic Arabidopsis plants, and two met this objective, to varying degrees. The promoter of *Gamete Expressed 1* (*AtGEX1*; *At5g55490*) directed expression in some somatic tissues, but in pollen it directed eGFP expression only in the sperm cells of tricellular pollen and not in the progenitor generative cell or in the vegetative cell. The *AtGEX2* promoter (*At5g49150*) directed eGFP expression in the generative cells of bicellular pollen and in the sperm cells in tricellular pollen grains, but not in other tissues; this pattern was similar to that seen with the *LGC1* and the *DUO1* promoters. We show that these promoters are useful for imaging sperm dynamics. The *AtGEX1* and *AtGEX2* promoters will be useful for manipulating gene expression in sperm, via antisense or overexpression constructs, to test whether candidate proteins play critical roles.

RESULTS

RT-PCR to Identify Possible Sperm-Specific Promoters

We previously reported the analysis of eight maize sperm transcripts that had similarity to hypothetical or

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unknown Arabidopsis proteins, and demonstrated by in situ hybridization that one of these transcripts was sperm specific in the mature pollen grain (Engel et al., 2003). Here, we used reverse transcription (RT)-PCR analysis to test an additional 155 transcripts from the maize sperm ESTs for their expression patterns in maize. We focused on transcripts whose deduced amino acid sequences were similar to hypothetical Arabidopsis proteins. Sperm-specific transcripts are probably poorly represented in cDNA libraries prepared from inflorescences or flowers; we therefore reasoned that genes without EST support in Arabidopsis might be sperm specific. We also tested transcripts that were present several times in the maize sperm EST database but were not represented in other maize EST databases. RT-PCR was carried out on random-primed RNA from mature pollen, unpollinated ear, unpollinated silks, seedlings, and root. We identified 35 transcripts that were expressed primarily in pollen (Supplemental Fig. 1; data not shown). These 35 transcripts were examined further by RT-PCR analysis using RNA prepared from different developmental stages, i.e. isolated unicellular microspores, bicellular and tricellular pollen grains, and fluorescence-activated cell (FAC)-sorted sperm cells. We identified 17 transcripts that appeared to be sperm specific in mature pollen grains or were expressed primarily in isolated sperm cells (Supplemental Fig. 1; Engel et al., 2003).

While maize is an excellent system for gamete isolation, it is not easy to transform. We were interested in examining gamete gene expression in a more easily manipulated plant. We started with the maize ESTs that appeared to be sperm specific (Engel et al., 2003; Supplemental Fig. 1) and then identified Arabidopsis genes whose deduced protein sequences were the best amino acid match to the deduced protein sequences of these maize ESTs. Occasionally, the maize ESTs had limited coding sequence, so we first identified a similar gene from rice (*Oryza sativa*) and then used the rice gene to identify the most similar Arabidopsis gene. This was particularly important for transcripts such as *Zmsp943* where most of the maize sperm EST was from the 3′ untranslated region (3′UTR). An Arabidopsis gene without EST support could not be found for all of the maize transcripts, but we identified 24 Arabidopsis genes for testing; in some cases, two Arabidopsis genes that were both similar to one maize transcript were tested.

We determined the expression patterns for these Arabidopsis genes using RT-PCR. Figure 1 shows the analysis of the five transcripts that appeared to be present predominately in RNA isolated from bud, flower, or pollen, while Supplemental Figure 1 shows the analysis of the other transcripts. *At1g66770* and *At5g62580* are both similar to *Zmsp041*. The *Zmsp041* transcript was shown to be present in sperm cells by in situ hybridization on pollen grains (Engel et al., 2003). The *At1g66770* transcript was present primarily in unopened buds, while the *At5g62580* transcript was present in mature pollen, unopened buds, open flower,

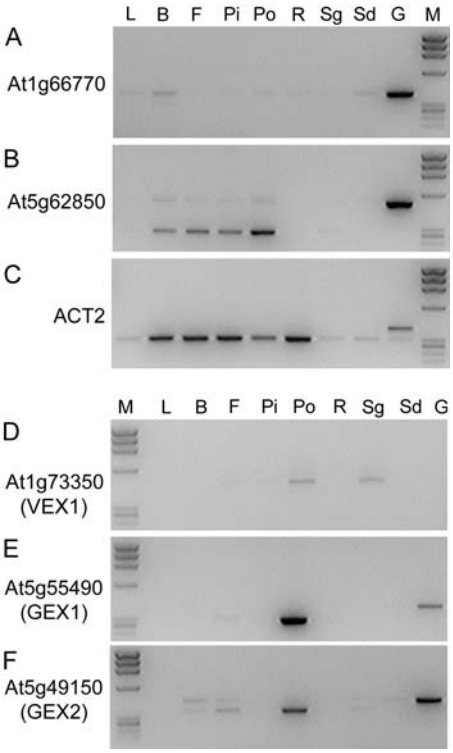

Figure 1. RT-PCR expression analysis of Arabidopsis transcripts. Gene-specific primers were used on random-primed cDNA. Each gene number is listed beside each gel. L, Leaf; B, flower bud; F, open flower; Pi, pistil; Po, pollen; R, root; Sg, seedling; Sd, seed; G, genomic DNA; M, marker ΦX174 digested with *Hae*III.

pistils, and detectable in leaves. *At1g73350* is similar to *Zmsp271*; it was present in pollen and seedlings. The *At5g55490* transcript is similar to *Zmsp943* and was present in mature pollen and flowers. The *At5g49150* transcript is similar to a contig composed of *Zmsp8028*, *Zmsp7334*, *Zmsp10477*, and *Zmsp7521* and was present in mature pollen, buds, and open flowers. The expression patterns for *Zmsp041*, *Zmsp271*, and *Zmsp943* in maize were reported (Engel et al., 2003). Briefly, *Zmsp041* was present in all stages of pollen development and also showed some expression in silks. *Zmsp943* was present primarily in isolated FACs-sorted sperm cells and was detectable in mature pollen. The *Zmsp8028* transcript was strongly expressed in FACs-sorted sperm cells, but it was also present to a lesser extent in the unicellular and mature pollen samples (Supplemental Fig. 1).

Because these five Arabidopsis genes appeared to be expressed in pollen or unopened buds, we tested whether any of their promoters would confer expression in sperm cells in vivo. Accordingly, sequences (approximately 1–2 kb) upstream of the putative initiating Met for each gene were amplified by PCR and cloned in front of the eGFP coding region. Arabidopsis (ecotype Columbia) plants were transformed, and the primary transformants were examined for eGFP expression during pollen development. Numerous plants containing the *At1g66770*::*eGFP* and *At1g73350*::*eGFP* constructs showed no eGFP expression in any tissue

examined (data not shown).This was unexpected because *At1g66770* was expressed in buds and *At1g73350* was expressed in pollen (Fig. 1A); perhaps the promoter fragments used were not long enough or the expression level was too weak. Nonetheless, Figure 2 shows that the other three promoters tested did express eGFP in the pollen grain (Fig. 2, G, M, and S) and that two (*At5g55490* and *At5g49150*) directed expression of eGFP in the sperm cells (Fig. 2, G and M). We named these two genes *AtGEX1* (*At5g55490*) and *AtGEX2* (*At5g49150*). The *Vegetative Cell Expressed 1* (*AtVEX1*; *At5g62580*) promoter was vegetative cell specific in pollen (Fig. 2S).

Developmental Expression of the Promoter-Reporter Gene Constructs

In tricellular pollen, both the *AtGEX1*::*eGFP* and *AtGEX2*::*eGFP* transgenic lines expressed eGFP in the sperm cells. We wanted to determine whether the promoters were active only in the sperm cells or if transcription was initiated earlier in pollen development.

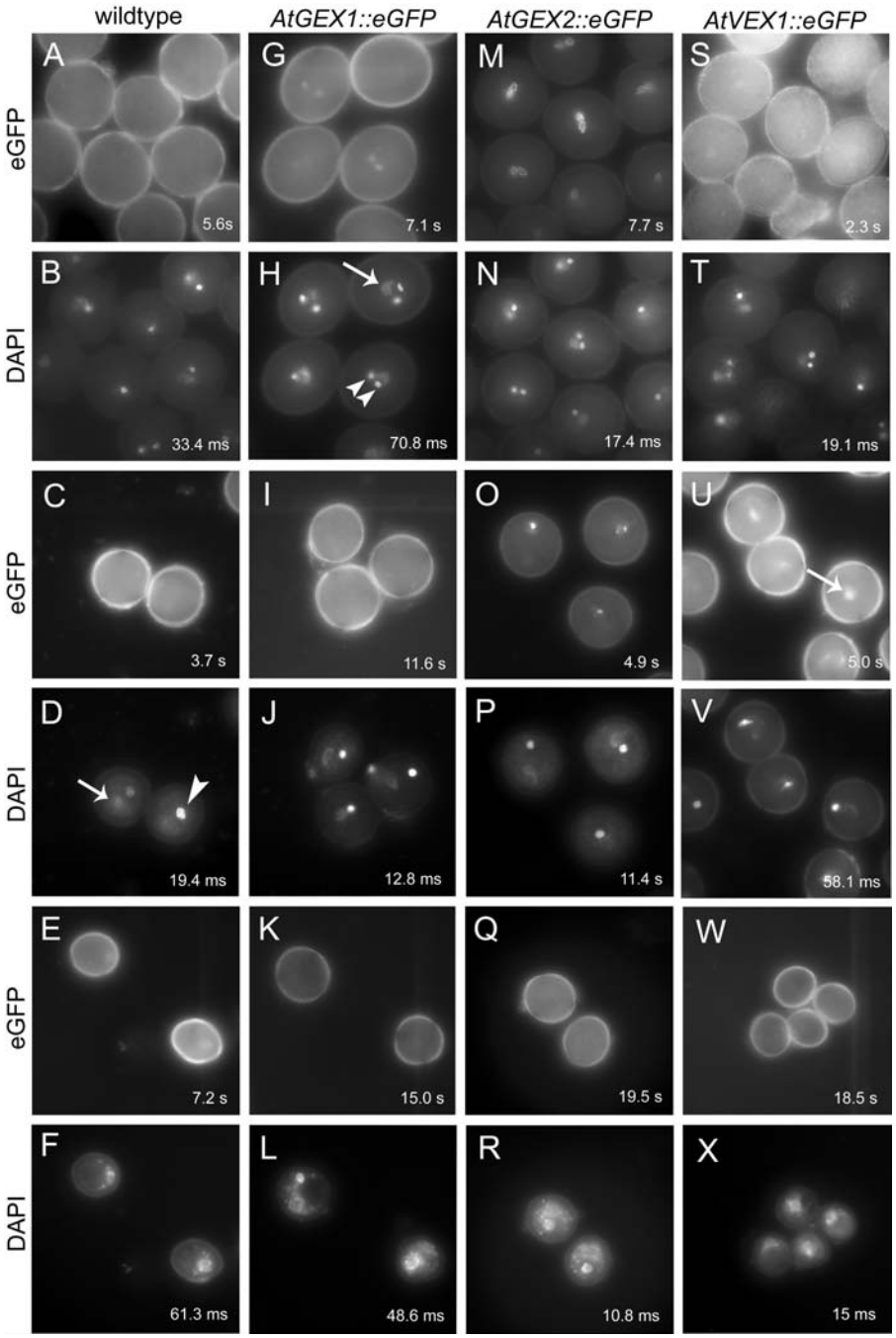

Figure 2. In vivo analysis of *AtGEX1*, *AtGEX2*, and *AtVEX1* promoters by *eGFP* expression during pollen development. This figure is arranged by developmental stage from top to bottom: mature pollen, bicellular pollen, and unicellular pollen. Fluorescence and *eGFP* expression is shown in paired rows that correspond to each developmental stage. In D, H, and U, the white arrow points to the vegetative nucleus. In D, the white arrowhead points to the generative nucleus. In H, the white arrowheads point to the sperm nuclei. The values in the lower right corners of each image represent the time of exposure for each image.

This question was relevant because by RT-PCR analysis we had found that a number of the maize sperm transcripts that were expressed in the sperm cells were also present at the unicellular and bicellular stages of pollen development (Engel et al., 2003). To examine the developmental pattern of expression, anthers were dissected, the pollen was stained with 4′,6-diaminophenylindole (DAPI) to determine the developmental stage, and then the pollen was examined for eGFP expression. In pollen, the *AtGEX1* promoter is only active in mature sperm cells (Fig. 2, G, eGFP, and H, DAPI) and not in bicellular pollen (Fig. 2, I and J) or unicellular pollen (Fig. 2, K and L). Because the *AtGEX1* promoter is not active in earlier stages of development, these results suggest that transcription and translation of the transgene must occur in the sperm cells. The *AtGEX1* promoter also drives expression of eGFP in the ovules, roots, and guard cells (data not shown).

In plants with the *AtGEX2*::*eGFP* construct, expression of eGFP in sperm cells was seen (Fig. 2, M, eGFP, and N, DAPI), but this promoter apparently initiates expression at the bicellular stage of pollen development because eGFP expression was also seen in generative cells (Fig. 2O). DAPI staining (Fig. 2P) showed two nuclei, one brightly staining (the generative cell) and one diffusely staining (the vegetative cell). For both constructs, some green fluorescence was seen at an earlier stage of pollen development. We believe this is autofluorescence and not eGFP because the fluorescence is present in all of the pollen grains (Fig. 2, Q and P, *AtGEX2*::*eGFP*; Fig. 2, I and K, *AtGEX1*::*eGFP*), even in heterozygous plants, and, more importantly, it is present in wild-type plants (Fig. 2, C and E). *AtGEX2*::*eGFP* is not expressed in any other tissues. The *AtVEX1* promoter was active in the vegetative cell of the pollen grain in tricellular pollen grains (Fig. 2, S, eGFP, and T, DAPI) and weakly active in bicellular pollen grains (Fig. 2, U and V); however, eGFP expression was not observed in unicellular pollen (Fig. 2, W and X).

In addition to the spatial variations observed with the *AtGEX1*::*eGFP* and *AtGEX2*::*eGFP* constructs, we occasionally observed a low level of greenish fluorescence in the vegetative cell of mature pollen; this expression appeared in addition to the sperm cell expression (Fig. 3C). The pollen grains shown in Figure 3, A and C, are from different flowers on the same *AtGEX1*::*eGFP* plant examined on the same day. The *AtGEX1*::*eGFP* construct only contained the putative promoter region, so we tested if the 3′UTR of the *AtGEX1* gene would affect the expression or translation of eGFP in the pollen grains. However, eGFP expression in plants transformed with a construct containing the 3′UTR (*AtGEX1*::*eGFPb*) was not appreciably different from that in plants expressing eGFP under the control of the 5′ promoter region alone (data not shown). We saw the same sporadic fluorescence in *AtGEX2*::*eGFP* plants; Figure 3, B and D, shows *AtGEX2*::*eGFP* pollen from the same plant examined

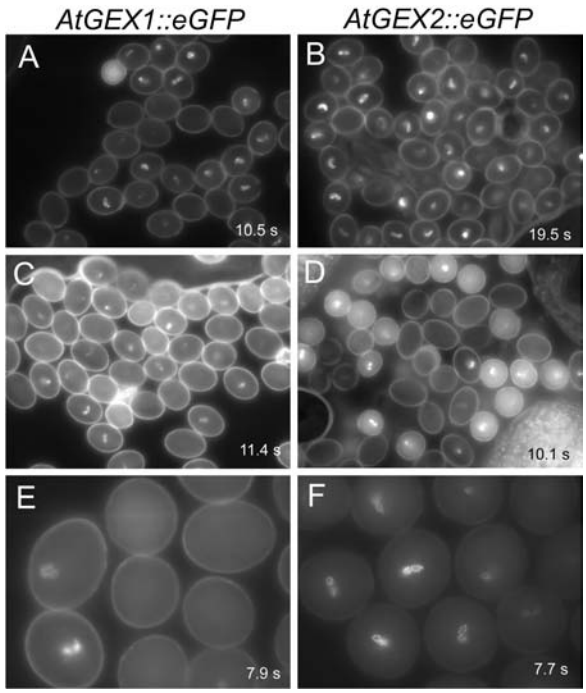

Figure 3. Variation in the *AtGEX1*::*eGFP* and *AtGEX2*::*eGFP* phenotypes. A, A field of pollen from a homozygous *AtGEX1*::*eGFP* plant. B, A field of pollen from a homozygous *AtGEX2*::*eGFP* plant. Note that all sperm cells are not in the same focal plane. C and D, Pollen from *AtGEX1*::*eGFP* (homozygous) and *AtGEX2*::*eGFP* (heterozygous) plants, respectively, exhibiting stochastic vegetative fluorescence. E and F, Pollen from homozygous *AtGEX1*::*eGFP* and homozygous *AtGEX2*::*eGFP* plants, under increased magnification. Note the presence of eGFP-expressing sperm cells in the *AtGEX2*::*eGFP* pollen grains and the absence of eGFP-expressing sperm cells in many of the *AtGEX1*::*eGFP* pollen grains.

on different days. Over the course of several months, we examined whether developmental stage, time of collection, heat treatment, or repeated desiccation influenced this greenish fluorescence in the vegetative cell, but none of these factors correlated with it. Pollen from wild-type plants grown side by side with the *AtGEX1*::*eGFP* or *AtGEX2*::*eGFP* plants occasionally exhibited greenish fluorescence (Fig. 2, A and C); thus, the sporadic fluorescence in the vegetative cell is not true expression of the transgene from the *AtGEX1* or *AtGEX2* promoters but is autofluorescence.

The *AtGEX1* promoter has lower expression levels overall than the *AtGEX2* promoter (Fig. 2, G versus M) and has variable expression, even in homozygous lines. This is most obvious in a *quartet* background, where all of the pollen grains from one meiosis remain attached to each other (Preuss et al., 1994). Figure 4 shows pollen from *AtGEX1*::*eGFP* and *AtGEX2*::*eGFP* plants in the *quartet* background. Figure 4A shows quartets from a plant that is heterozygous for the *AtGEX1*::*eGFP* transgene; sperm cells are visible in only one or two pollen grains in the quartet. The variable expression is also present in plants homozygous for the *AtGEX1*::*eGFP* transgene.

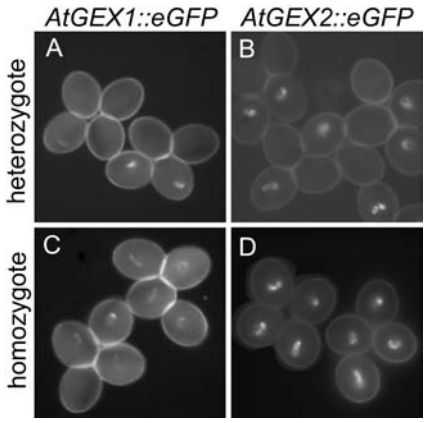

Figure 4. *AtGEX1* and *AtGEX2* promoters driving eGFP expression in the *quartet1* background. A, Pollen from a plant heterozygous for *AtGEX1*::*eGFP*. B, Pollen from a plant heterozygous for *AtGEX2*::*eGFP*. C, Pollen from a plant homozygous for *AtGEX1*::*eGFP*. D, Pollen from a plant homozygous for the *AtGEX2*::*eGFP*.

Figure 4C shows two quartets: in one, sperm cells are visible in all four pollen grains, as expected, while in the other sperm cells are only visible in three pollen grains. By contrast, the *AtGEX2*::*eGFP* expression is fully penetrant: The heterozygous plants always have two pollen grains with green sperm in each quartet (Fig. 4B), while in homozygous plants all of the pollen grains have eGFP expression in the sperm cells (Fig. 4D). These patterns of expression were consistent in the six independent *AtGEX2*::*eGFP* lines and in the more than 30 independent *AtGEX1*::*eGFP* lines we analyzed, suggesting that the expression differences between the promoters are not the result of position effects.

Live Imaging of Sperm Cell Dynamics

Transmission electron microscopy studies of sperm cells have revealed the subcellular organization of the sperm cells and their close association with the vegetative cell nucleus (for review, see Dumas and Mogensen, 1993; Weterings and Russell, 2004). However, such studies give only a static picture. By contrast, expression of *AtGEX1*::*eGFP* and *AtGEX2*::*eGFP* allows imaging of live sperm cells over time and reveals that the sperm cells are highly dynamic. In living cells when the *LAT52* promoter (Twell et al., 1990) is used to express eGFP, it is possible to see sperm shadows within the fluorescently green vegetative cell (Fig. 5A). The expression of eGFP in the cytoplasm of the sperm cells allows visualization of sperm morphology that is significantly superior to that possible after DAPI staining of sperm nuclei (Fig. 2, B, H, N, and T; Lalanne and Twell, 2002) or with nuclear-targeted fluorescent protein markers (Durbarry et al., 2005; Rotman et al., 2005). Figure 5B shows cytoplasmic eGFP expression in sperm cells. Figure 5C is the same pollen grain stained with DAPI so that the sperm nuclei are visible. The superimposition of these images (Fig. 5D) clearly illustrates the differences between imaging the cytoplasm and the nuclei of the sperm cells. Figure 5, E to T, shows examples (with *AtGEX2*::*eGFP* pollen) of the vastly different morphologies of the sperm cells in vivo; similar images are obtained with *AtGEX1*::*eGFP* pollen. Note the tail-like cytoplasmic extension of the sperm cells (Fig. 5, E and P) as well as the nuclear shadows within the sperm cells (Fig. 5, K, L, and T). These images contradict the common perception that sperm cells are largely a nucleus with minimal cytoplasm. Unlike the sperm cells in vivo, sperm cells released from broken pollen grains are round (Fig. 5, U and V; see also Engel et al., 2003). Supplemental Figure 2, A to D, are time-lapse (images acquired over 6–30 min) movies. The movement of sperm is highly dynamic as the cells can be seen going up and down in the pollen tube and moving around within the pollen grain before entering the tube. The cytoplasmic extensions of the two sperm cells can be seen as well as their interaction with the vegetative nucleus.

Analysis of Promoter Sequences

Genes with common patterns of expression frequently have common sequence motifs in their promoters. We wanted to determine if there were any common sequence motifs that might be responsible for sperm cell expression. We compared *AtGEX1* and *AtGEX2* to *LGC1* from lily, to the putative maize *ZmGEX1* promoter, to the putative rice *OsGEX1* and *OsGEX2* promoters, and to the putative poplar (*Populus trichocarpa*) *PtGEX1* and *PtGEX2* promoters. The putative *ZmGEX1* promoter was identified using the BLAST function at the Maize Genome Assembly Project (http://maize.ece.iastate.edu/magi.html). The *Zmsp943* sequence was used to identify a Maize Assembled Genomic Island (MAGI) that contained the same sequence. Successive searches using each identified MAGI to find an overlapping MAGI allowed us to isolate the entire coding region of the *ZmGEX1* gene and 1.4 kb upstream of the predicted initiating Met. We called the region upstream of the predicted initiating Met the putative promoter. There currently is no MAGI that contains the initiating Met of the *ZmGEX2* gene, so we were unable to examine the putative promoter region from this gene. We used a database search to identify the rice proteins that were most similar to the AtGEX1 and AtGEX2 proteins. We then analyzed the 2-kb regions upstream of the initiating Met of these rice proteins.

It has been reported (Singh et al., 2003) that the *LGC1* promoter has a TATA box and a GC box, but none of the other promoters examined had a GC box. The *LGC1* promoter also has a region similar to a G or T box with a core ACGT motif; this motif is often the target of bZIP binding proteins. Supplemental Figure 3 illustrates the relative location of the promoter elements identified in the *GEX1* and *GEX2* promoters. The *AtGEX1* and *ZmGEX1* promoters have an obvious TATA box and a CCAAT box, but the *OsGEX1* and the

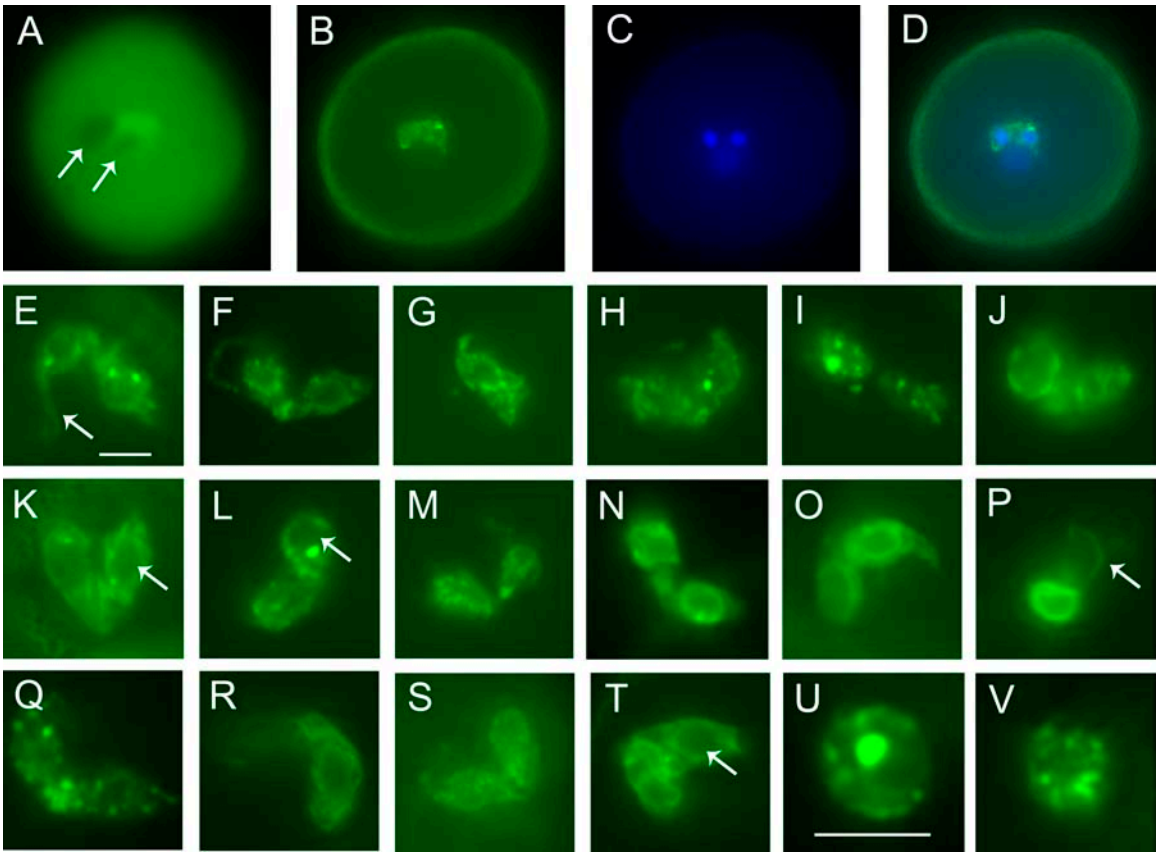

Figure 5. Visualization of sperm cells. A, The white arrows point to sperm shadows in LAT52::eGFP pollen. B, eGFP expression in *AtGEX2*::*eGFP* sperm cells. C, DAPI staining of the vegetative and sperm nuclei in the same pollen grain as in B. D, An overlay of sections B and C. E to T, In vivo sperm cell morphologies observed in individual *AtGEX2*::*eGFP* pollen grains. In E and P, the white arrows point to the cytoplasmic tail of the sperm cells. In K and L, the white arrows point to the nucleus. In E, the white bar represents 4 microns. U and V, *AtGEX2*::*eGFP* sperm cells floating free in solution. In U, the white bar represents 2 microns.

PtGEX1 putative promoters do not have either. The *AtGEX2* promoter does not have an obvious TATA box but does have a canonical CCAAT box, whereas the *PtGEX2* putative promoter has a TATA box but no CCAAT box. The *OsGEX2* putative promoter lacks both a TATA and a CCAAT box. A number of testis-specific promoters from mammals are TATA-less (Kaneko and DePamphilis, 2000; Iguchi et al., 2004). Like *LGC1*, the *AtGEX2* promoter has three core ACGT binding sites; the *OsGEX2-japonica* putative promoter contains four of these core sites, while the *OsGEX2-indica* putative promoter has only two. *PtGEX2* has no ACGT binding sites. All of the *GEX1* promoter regions also contain ACGT core motifs: *ZmGEX1* has four, *OsGEX1-japonica* has one, *OsGEX1-indica* has two, *PtGEX1* has six, and *AtGEX1* has five.

G boxes were found in the *AtGEX2* promoter and in both *OsGEX2* (japonica and indica cultivars) putative promoters. A G-box binding protein transcript was present several times in the maize sperm EST sequences (Engel et al., 2003). Additionally, all of the *GEX2* promoters contained at least one AAAG sequence, which is the core binding site for Dof transcription factors (Yanagisawa and Schmidt, 1999). Interestingly, the *AtGEX2* promoter and the putative promoters of O*sGEX2-japonica*, *OsGEX2-indica*, and *PtGEX2* each have an AAAG sequence located between 333 bp and 342 bp upstream of the translational start site. The *AtGEX1* and the *OsGEX1-japonica* and *OsGEX1-indica* promoters also have an AAAG sequence located within this same region; however, in the *ZmGEX1* and *PtGEX1* promoters, the AAAG is located closer to the translational start site. The *LGC1* promoter also contained two of these motifs, but they are outside of the region required for generative cell-specific expression (Singh et al., 2003). Dof transcription factors are known to interact with bZIP binding proteins (Zhang et al., 1993; Vicente-Carbajosa et al., 1997). No other common known motifs could be found among all of the promoters using visual inspection or the MatInspector program from GenomatixSuite (Quandt et al., 1995) with the plant cis-acting regulatory DNA elements database (Higo et al., 1999).

We also compared the promoters of *AtGEX1* and *AtGEX2* to two other promoters known to drive expression in sperm cells, *DUO1* and *ANTIKEVORKIAN* (Rotman et al., 2005). *AtGEX1*, *AtGEX2*, *DUO1*, and *ANTIKEVORKIAN* all had an $AAG^{T}/_{A}TC$ motif,

located approximately 300 bp upstream of the translational start site; however, this consensus was not found in the rice, maize, or poplar *GEX* promoters.

AtGEX1 and AtGEX2 Protein Analysis

An alignment of the rice, maize, sorghum (*Sorghum bicolor*), poplar, and Arabidopsis GEX1 proteins is shown in Supplemental Figure 4, and an alignment of the partial ZmGEX2 protein sequence and the rice (japonica and indica cultivars), poplar, and Arabidopsis GEX2 proteins is shown in Supplemental Figure 5. We used the BLASTP program to search for conserved domains in the GEX1 and GEX2 proteins, but we were unable to find any significant matches, i.e. e-values of 1e−8 or smaller.

The TMPred (Prediction of Transmembrane Regions and Orientation) program (http://www.ch.embnet.org/software/TMPRED_form.html) predicts that *AtGEX1* encodes a protein with three transmembrane domains. The PSORT program (http://psort.nibb.ac.jp) predicts a cleavable signal sequence of 24 amino acids. This predicted signal sequence is not included in the annotated version of At5g55490 (The Arabidopsis Information Resource [TAIR] and the National Center for Biotechnology Information [NCBI]) but is in frame with an upstream Met in the genomic sequence. The annotated OsGEX1 protein (NCBI) includes this region, which encodes a predicted signal sequence. The *ZmGEX1* and the *PtGEX1* genes also have a predicted signal sequence. Since all four proteins appear to have a similar structure, we believe that the Arabidopsis protein is misannotated. We isolated the *AtGEX1* coding region; with the exception of a single amino acid change, it does not differ from the protein sequence predicted in the database. TMPred predicts that ZmGEX1, OsGEX1 (japonica and indica cultivars), PtGEX1 (poplar), and SbGEX1 (sorghum) have three transmembrane domains.

AtGEX2 is predicted to have six transmembrane domains, but is not predicted to have a cleavable signal sequence. The *AtGEX2* coding region we isolated has a splice site change from the annotated protein sequence (TAIR, NCBI) that removes 11 amino acids; these 11 amino acids are not present in the rice and poplar GEX2 proteins. The presence or absence of these 11 amino acids does not affect the prediction of the transmembrane domains. The OsGEX2-japonica and OsGEX2-indica proteins are also predicted to have six transmembrane domains. They also have predicted cleavable signal sequences by PSORT; however, they have approximately 140 additional N-terminal amino acids, and, for both proteins, there is a Met present after the predicted signal sequence. Thus, these proteins maybe misannotated. PtGEX2 is also predicted to have six transmembrane domains; however, because of the uncertainty of the initiating Met, we cannot determine whether it has a cleavable signal sequence. We have not isolated the full-length *ZmGEX2* coding region.

Proteins involved in sperm-egg or sperm-central cell interactions are likely to be secreted or plasma membrane bound. *AtGEX1* and *AtGEX2* are expressed in sperm, and the proteins encoded by them are both predicted to encode plasma membrane proteins. To determine if AtGEX1 and AtGEX2 are plasma membrane bound, we examined their expression as N-terminal fusion proteins with eGFP, under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The constructs were bombarded into onion (*Allium cepa*) epidermal peels. Figure 6A shows an onion cell expressing the AtGEX1-eGFP fusion protein; the eGFP signal is located primarily in the margins of the cell. When these onion peels were plasmolyzed by treating with 0.75 M sodium chloride, according to the protocol of Guyon et al. (2004), the eGFP signal remained associated with the membrane (Fig. 6, B, eGFP, and C, bright field of same cell). The AtGEX2-eGFP fusion protein behaved in a similar manner: Figure 6D shows the cells before salt treatment, and Figure 6, E and F, shows the cells after salt treatment. The eGFP control was expressed at a higher level and was present predominantly in the cytoplasm (Fig. 6G). More fluorescence was seen in the cytoplasm after salt treatment (Fig. 6H) in the eGFP control than with either of the fusion proteins.

DISCUSSION

We have used maize sperm ESTs to identify three Arabidopsis promoters that are active in pollen. In

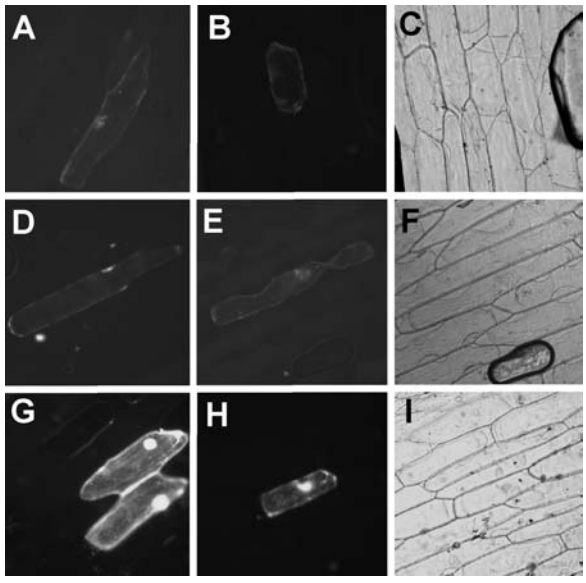

Figure 6. Transient expression of CaMV*35S*::*AtGEX1-eGFP* and CaMV*35S*::*AtGEX2-eGFP* constructs. A to C, Onion epidermal cells expressing the AtGEX1-eGFP fusion protein. B, Cells were plasmolyzed by salt treatment. C, Bright-field illumination of the same cells as in B. D to F, Onion epidermal cells expressing the AtGEX2-eGFP fusion protein. E, Cells were plasmolyzed by salt treatment. F, Same cells as in E, bright-field illumination. G to I, Onion epidermal cells expressing eGFP protein alone. H, Cells were plasmolyzed by salt treatment. I, Same cells as in H, bright-field illumination.

microarray experiments (AtGenExpress, 2004; Craigon et al., 2004), the *AtGEX1* and *AtGEX2* transcripts are called present in pollen. Because there is no a priori way to identify those genes that might be sperm specific from microarray experiments with pollen RNA, our approach, to test genes corresponding to transcripts first identified as sperm specific in maize, was reasonable. It is notable that sequences similar to *AtGEX1* and *AtGEX2* (Supplemental Figs. 4 and 5) are found only in genomic DNA, from plants that have been fully or partially sequenced, and are essentially not represented in the extensive plant EST databases. Testing promoters of genes whose transcripts are present in pollen RNA samples but underrepresented in EST databases is a promising strategy by which to identify additional sperm-specific genes. The GEX1 and GEX2 proteins are predicted to be in the plasma membrane, and transient expression in onion epidermal cells (Fig. 6) supports that localization. Except for the transmembrane domains, neither protein has recognizable motifs, so the roles these proteins play in sperm are unknown. Antisense or RNAi approaches, using sperm-expressed promoters, might reveal functions for GEX1, GEX2, and other sperm proteins.

Promoter Reliability

We set out to identify promoters that could be used to express reporter or other transgenes specifically and consistently in male gametes. How reliable are the *AtGEX1* and *AtGEX2* promoters for these purposes? Expression from the *AtGEX2* promoter was more robust than from the *AtGEX1* promoter. *AtGEX2* can drive expression of transgenes in both the generative cell and the sperm cells. Whether this is a result of transcription or translation in the sperm cells or of partitioning of transcripts or protein from the generative cell is unknown. Expression from the *AtGEX1* promoter is much weaker than that from the *AtGEX2* promoter. Perhaps the threshold needed for eGFP fluorescence to be visualized is not reached in all pollen grains, resulting in stochastic gene expression. It is known that expression levels can be changed by small changes in promoter sequence, which indicates that different promoters have different levels of variability (Raser and O'Shea, 2004). The variability of gene expression is also dependent on the number of copies of a gene: Fewer copy genes show more variation (Raser and O'Shea, 2004), suggesting that expression in haploid cells like sperm might be more variable than in a diploid somatic cell.

Because the *AtGEX2* promoter has a higher level of expression and more reproducible expression in all pollen grains, it will be the most useful for future expression studies. In pollen, the *AtGEX1* promoter-reporter gene construct directed gene expression only in sperm cells and not in the progenitor generative cell, but weak expression was noticed in ovules, roots, and guard cells. Therefore the *AtGEX1* promoter is not as useful. However, analysis of the *AtGEX1* promoter-reporter gene construct did allow us to answer whether sperm are transcriptionally and translationally competent. Because some sperm-specific transcripts were present in earlier stages of pollen development in maize (Engel et al., 2003), it was formally possible that sperm cells were largely transcriptionally and translationally quiescent, or that they were only capable of translation. The promoter activities of the *LGC1* and *DUO1* genes cannot resolve this possibility since they are also expressed (Singh et al., 2003; Durbarry et al., 2005) in the progenitor generative cell. The activity of the *AtGEX1* promoter in sperm cells in mature pollen conclusively proves that sperm cells are capable of both de novo transcription and translation.

The *AtGEX2* promoter drives expression similar to that of the *LGC1* promoter from lily and the Arabidopsis *DUO1* promoter, while the *AtGEX1* promoter is expressed later. Because *AtGEX1* and *AtGEX2* are expressed at different developmental stages, it is likely that they have differential transcriptional regulation. Even when promoters drive similar patterns of gene expression, they may not have significant sequence similarity, as is the case for the extensively characterized pollen promoters *LAT52* and *LAT59* (Twell et al., 1991; Eyal et al., 1995). Whether any of the predicted transcription factor binding sites in the *AtGEX1* and *AtGEX2* promoters (Supplemental Fig. 3) are important for generative cell or sperm cell expression remains to be determined. The generative and sperm cell-specific expression pattern directed by the *LGC1* promoter is thought to be due to a repression element because deletion constructs of the *LGC1* promoter exhibited vegetative cell and somatic cell expression of the reporter gene (Singh et al., 2003). It will be interesting to see if the *AtGEX2* and *DUO1* promoters also have a repression element (Singh et al., 2003) or if the genes are regulated differently.

Imaging and Other Uses for These Promoters

Expression of eGFP in the sperm cells will allow for improved imaging of sperm dynamics under both in vivo and in vitro conditions (Fig. 5; Supplemental Fig. 2, A–D). Strongly expressed eGFP frequently concentrates in the nuclei of cells (e.g. Fig. 5A). The absence of eGFP in the sperm nuclei (e.g. Fig. 5K) is likely due to the relatively weaker expression from the *AtGEX* promoters, given that a nuclearly targeted red fluorescent protein was able to mark sperm nuclei (Rotman et al., 2005). We can anticipate multicolored pollen grains, with the sperm cytoplasm, the sperm nuclei, the vegetative nucleus, and the actin cytoskeleton (Sheahan et al., 2004) of the vegetative cell each marked with different fluorescent proteins. Such transgenic lines and live cell imaging would help define the interactions of the sperm cells with the vegetative nucleus and the actin cytoskeleton as the sperm cells progress down the pollen tube. These transgenic lines should also facilitate screens for mutants with altered sperm positions (e.g. Lalanne and Twell, 2002) or

dynamics. In addition to expression in sperm cells, we occasionally saw autofluorescence in the vegetative cell (Fig. 3, C and D), but were unable to determine its cause. Researchers planning to use these promoters should be aware of this issue, but even if such autofluorescence is seen in some pollen grains, it does not appreciably interfere with visualization of the sperm cells in other pollen grains.

Although we did not set out to find a late-stage vegetative cell promoter, the *AtVEX1* promoter should prove useful. Most promoters used for expression of transgenes in pollen, such as *LAT52* (Twell et al., 1990), are active in the vegetative cell at early stages. Early expression of transgenes is not always desirable. For example, Lee et al. (1996) could not determine if the pollen receptor kinase PRK1 was involved in pollen germination because the antisense construct with the *LAT52* promoter resulted in pollen abortion at the unicellular microspore stage.

The *AtGEX1* and *ATGEX2* promoters can be used to examine the function of sperm proteins thought to be involved in fertilization, by using them to express GFP-fusion proteins to examine protein localization, or to express antisense or overexpression constructs. It is likely that the *GEX1* and *GEX2* genes will have similar expression patterns in other organisms, and so these promoters or their homologs should prove useful for similar studies in a variety of plant species. Finally, GFP-tagged sperm cells (Fig. 5, U and V), combined with FACS sorting (Engel et al., 2003), should allow isolation of large numbers of Arabidopsis sperm cells. RNA prepared from FACs-purified Arabidopsis sperm could be hybridized to whole-genome microarrays to determine the transcriptome of sperm cells. The availability of the sequenced genome and mass spectroscopy to identify proteins will provide a more comprehensive way of determining what proteins are present in the sperm cell.

MATERIALS AND METHODS

RT-PCR

RNA was extracted from Arabidopsis (*Arabidopsis thaliana*) leaves, buds, flowers, pistils, and pollen using the RNeasy Kit (Qiagen, Valencia, CA), and from roots, seedlings, and seeds according to Scott (1995). Unicellular microspores and bicellular pollen were collected, and all *Zea mays* RNA was isolated as described (Bedinger and Edgerton, 1990). For the Arabidopsis expression analysis, 2 μg of total RNA was reverse-transcribed using the SuperScript II system (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, with random hexamers in a 20-μL reaction. PCR was carried out using 0.5 μL of the RT reaction in 25-μL reactions (0.2 mM dNTPs, 0.2 μM forward (F) primer, 0.2 μM reverse (R) primer, 1× Taq buffer, and 2.5 units Taq polymerase (New England Biolabs, Beverly, MA), using these conditions: 5 min at 95°C; 30 cycles at 30 s at 95°C, 30 s at 60°C, 75 s at 72°C; and 10 min at 72°C, except for *At1g73350*, for which primer annealing was done at 62°C. The primers used for PCR are as follows: At1g73350 F, 5′TTGCTGGTGAACGTGGAGGC3′, and R, 5′CCCAACAAGCCTTTCGGAGC3′; At1g66770 F, 5′TCCTGATAGCACCTTGCTCG3′, and R, 5′GGTATAGCCAGGAATGGATC3′; At5g62850 F, 5′ACTTCCTTTCGTCCAGCC3′, and R, 5′ATGACGGTGAGAGGAGC3′; At5g55490 F, 5′ACCGGCGTTTCCAACCTG3′, and R, 5′GTTTGTCACACTAGCCACG5′; At5g49150 F, 5′CAAACATTGAATGGTGGTCC3′, and R, 5′ATGGCATACTAGAGATGCTC3′; and ACTIN2 F, 5′GCCATCCAAGCTGTTCTCTC3′, and R, 5′TTCTCGATGGAAGAGCTGGT3′. Thirty cycles of PCR was determined to be within the linear range of amplification for these conditions. Ten microliters of each PCR reaction was run on a 1.5% agarose gel and imaged. All images were reversed using Adobe Photoshop Elements version 2.0 (Adobe Systems, Mountain View, CA).

Promoter Isolation and Plant Transformation

The putative promoters were amplified from Arabidopsis ecotype Columbia genomic DNA, using the following primer pairs: for the 1-kb putative promoter of *At1g73350*, F, 5′GATCGAAGCTTGAAGATTCATGTCACGTCTC3′, and R, 5′GATGGATCCGATTCTACCTCGCTTTAATCCCG3′; for the 1.8-kb putative promoter of *At1g66770*, F, 5′GATCGAAGCTTGACGCGAAGACGTGGTC3′, and R, 5′GATGGATCCGTTATGATGAAGGGTAGTTATC3′; for the 2.1-kb putative promoter of *At5g62850*, F, 5′CGGGATCCATATGTATAACCGTTGAATTTTC3′, and R, 5′TTCCCGGGCTAAAGAAGAATTCTTTATCTTC3′; for the 1.8-kb putative promoter of *At5g55490*, F, 5′GATCTCTAGAGCTACCTCAACGCACCTTG3′, and R, 5′GTAGCCGGGCCTTGTGATTGATCACCTAC3′; and for the 1.8-kb putative promoter of *At5g49150*, F, 5′GGTCTGAATTCTTACATCGGATGGATTCAC3′, and R, 5′CTAACACCCGGGTACATTAACCCTTCACAACAAG3′. The putative promoter fragments were cloned into pEGAD (Cutler et al., 2000) to yield *At1g73350::eGFP*, *At1g66770::eGFP*, *At5g62850::eGFP*, *At5g55490*::eGFP, and *At5g49150::eGFP*. An additional construct (*At5g55490::eGFPb*), which contained both the putative promoter region and the putative 3′UTR of the gene, was also constructed. The putative 3′UTR was amplified with F, 5′GATCTCTAGATAGCCACCAAAGTTTTTCAC3′, and R, 5′GATCTCTAGAATGATTCAGATCTGTCGATG3′.

Arabidopsis cv Columbia was transformed by Agrobacterium-mediated transformation (Clough and Bent, 1998). Seeds were plated on selection plates containing 7.5 μg mL^{-1} ammonium glufosinate (Sigma, St. Louis). Resistant seedlings were transferred to soil and grown in a greenhouse under a 16-h-day, 8-h-night light regimen with day temperatures of 20°C to 22°C and night temperatures of 16°C to 18°C and an average humidity of 55%. *AtGEX1::eGFPb* and *AtGEX2::eGFP* seeds are available from the Arabidopsis Biological Resource Center.

Arabidopsis Floral Developmental Series and Pollen Germination

Open flowers and closed buds were dissected and the anthers removed. Mature and immature pollen was dissected from the anthers and placed in 0.05 M $NaPO_4$, pH 7.0, and 0.5% Triton X-100 containing 1 μg/mL DAPI.

To study the dynamics of sperm cell movement in germinating pollen tubes, pollen was germinated on a Nucleopore membrane using the protocol of Johnson-Brousseau and McCormick (2004) and the pollen germination medium of Thorsness et al. (1993). Briefly, a piece of Nucleopore membrane was placed on a microscope slide and wetted with pollen germination medium. Anthers were dabbed onto the membrane to release the pollen. A drop of pollen germination medium was placed on a coverslip, which was inverted and then placed over the membrane. The coverslip was sealed with nail polish. Pollen tubes were observed within 30 min.

Microscopy

Pollen was examined with a Zeiss Axiovert 200 M microscope (Carl Zeiss, Jena, Germany), and images were captured using an AxioCamMR camera and AxioVision 4.3 software. The microscope was equipped with a GFP filter set comprised of an excitation filter (BP 470/20 nm), a beam splitter (495 nm), and an emission filter (LP 505–530 nm) and a DAPI filter set comprised of an excitation filter (BP 365/12 nm), a beam splitter (395 nm), and an emission filter (LP 397 nm). The objectives used for imaging were the Neofluar 40× oil, the Apochromat 63× oil, and the Neofluar 100× oil. The software and camera automatically determined optimal exposure time. The exposure time for each image is noted. The images were compiled using Adobe Photoshop Elements version 2.0 (Adobe Systems).

Transient Expression Assays

The full-length At5g55490 protein and the full-length At5g49150 protein were expressed as N-terminal fusions with eGFP under the control of the

CaMV35S promoter. The *AtGEX1* cDNA was isolated from Arabidopsis pollen RNA by using *At5g55490*Ra, 5′TAGCCGGATCCAATCTTCTAGTCAATGATGAAG3′, for first-strand synthesis and amplifying with *At5g55490*Ra and *At5g55490*Fa, 5′ATCCGAATTCATGGATCGTTTCAGCAG3′. The *AtGEX2* cDNA was isolated from Arabidopsis pollen using *At5g49150*Ra, 5′GCTAGGTACCACCTATCAGAACCATTAAC3′, for first-strand synthesis and amplifying the product with *At5g49150*Ra and *At5g49150*Fa, 5′CTAGTCTAGAATGTACCCATCTGTTAGTG3′. Both cDNAs were cloned into pCAMBIA-2300 (http://www.cambia.org/main/r_et_vman.htm) between the CaMV35S promoter and eGFP from the pEGAD vector. Onion (*Allium cepa*) epidermal cells were transformed by gold particle bombardment using a Bio-Rad (Hercules, CA) gene gun.

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or part of the material. Obtaining any permissions will be the responsibility of the requestor.

Note Added in Proof

Strompen et al. (2005) demonstrated that At3g08560 is sperm expressed. This promoter also has a Dof transcription factor binding site at −343 from the ATG.

Strompen G, Dettmer J, Stierhof Y-D, Schumacher K, Jurgens G, Mayer U (2005) Arabidopsis vacuolar H^+-ATPase subunit E isoform 1 is required for Golgi organization and vacuole function in embryogenesis. Plant J **41:** 125–132

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY746360 (*AtGEX1*) and AY746359 (*AtGEX2*).

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CORRECTION

Vol. 138: 2124–2133, 2005

Engel M.L., Holmes-Davis R., and McCormick S. Green Sperm. Identification of Male Gamete Promoters in Arabidopsis.

The locus tag for AtVex1 is incorrectly stated in the abstract and text, as it should be At5g62850, not At5g62580. Additionally, a 1.2-kb region upstream of At5g62850, not a 2.1-kb region, was used as the putative promoter, and this fragment omitted an approximately 250-bp region just upstream of the currently annotated initiating Met.