Intercellular communication in *Arabidopsis thaliana* pollen discovered via *AHG3* transcript movement from the vegetative cell to sperm

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Edited by June B. Nasrallah, Cornell University, Ithaca, NY, and approved September 23, 2015 (received for review June 4, 2015)

An *Arabidopsis* pollen grain (male gametophyte) consists of three cells: the vegetative cell, which forms the pollen tube, and two sperm cells enclosed within the vegetative cell. It is still unclear if there is intercellular communication between the vegetative cell and the sperm cells. Here we show that *ABA-hypersensitive germination3* (*AHG3*), encoding a protein phosphatase, is specifically transcribed in the vegetative cell but predominantly translated in sperm cells. We used a series of deletion constructs and promoter exchanges to document transport of *AHG3* transcripts from the vegetative cell to sperm and showed that their transport requires sequences in both the 5′ UTR and the coding region. Thus, in addition its known role in transporting sperm during pollen tube growth, the vegetative cell also contributes transcripts to the sperm cells.

pollen | sperm | mRNA transport | protein phosphatase 2C | vegetative nucleus

Pollen grains are derived by stereotypical cell divisions (1, 2). Each male meiotic product (microspore) undergoes an asymmetric mitotic division, which generates a bicellular pollen grain composed of a vegetative cell and a generative cell in which the generative cell is engulfed inside the cytoplasm of the vegetative cell. The generative cell undergoes a second mitosis to generate two sperm cells. The vegetative cell forms the pollen tube that delivers the sperm to the embryo sac. One sperm cell fertilizes the egg to produce the zygote, and the second sperm cell fuses with the central cell to produce the endosperm (3).

Intercellular communication plays an important role in the regulation of plant development (4). Plasmodesmata, microscopic channels that traverse the cell walls of most plant cells, are usually the conduit for intercellular transport in plants (5). Plant sperm are surrounded by their own plasma membrane and by an endomembrane of vegetative cell origin; there is a thin polysaccharide extracellular matrix between these two membranes, but there is no true cell wall comprised of cellulose and callose (6). Although pollen grains lack bona fide plasmodesmata, plasmodesmata-like connections between the sperm and vegetative cell cytoplasm were reported in *Nicotiana alata* pollen grains (6). In addition, there is a cytoplasmic projection that connects one sperm cell with the vegetative cell nucleus, first observed in cotton (7) and then described in other species (reviewed in ref. 2). Moreover, the two sperm cell membranes are connected to each other through a tetraspanin-enriched microdomain (8). Although all these physical connections presumably ensure that the vegetative nucleus and the sperm cells move in the pollen tube as a unit (known as the "male germ unit"), they also may provide a route for intercellular communication. It has been proposed that small RNAs move from the vegetative cell to sperm cells (9); however, this notion has been challenged (10). Moreover, the reported mechanism of mRNA movement and small RNA movement in sporophytic tissues is different (11, 12). Thus, to date there is no unequivocal evidence of intercellular mRNA communication between the vegetative cell and the sperm cells during pollen development.

In this study we investigated if there is transport between the vegetative cell and sperm cells. While studying *ABA-hypersensitive germination3* (*AHG3*), which encodes a protein phosphatase 2C, we unexpectedly found that the promoter of *AHG3* was transcriptionally active in the vegetative cell, whereas a translational fusion protein, AHG3-GFP, driven by the same *AHG3* promoter, was localized in sperm. These different localizations suggested that *AHG3* transcripts or the AHG3 protein could move from the vegetative cell to sperm cells. Here we provide evidence that *AHG3* transcripts move from the vegetative cell to sperm cells and that the transport of *AHG3* transcripts requires sequences in both the 5′ UTR and coding region. Our results thus document an additional role for the vegetative cell in providing transcripts to the sperm cells.

Results

The Pollen Transcription Pattern of *AHG3* Is Different from Its Protein Pattern. Protein phosphorylation and dephosphorylation are important mechanisms for modulating protein activity. In the course of experiments to study protein phosphorylation during pollen development, we became interested in a PP2C type of protein phosphatase, AHG3, whose transcripts accumulated in sperm cells (13). AHG3 is a negative regulator of the abscisic acid (ABA) pathway in sporophytic parts of the plant (14, 15). *AHG3* expression was ABA-inducible in roots, leaves, inflorescences, and siliques, as judged by Pro_{AHG3}:*GUS* lines (14). According to microarray analysis (13), the expression value for *AHG3* was about 10 times higher in sperm cells

Significance

In pollen grains, the known function of the vegetative cell is to extend a pollen tube to transport the two sperm cells to the embryo sac for fertilization. Because the vegetative cell is much larger and more metabolically active than sperm, it has been assumed that the vegetative cell also might supply various components to sperm, but there was no direct evidence to support this hypothesis. We used deletion and promoter exchange constructs to provide direct evidence that the vegetative cell provides protein-encoding transcripts to sperm cells, highlighting a previously unidentified role of the vegetative cell.

Author contributions: H.J., C.K., and S.M. designed research; H.J., J.Y., L.C.B., and Y.C. performed research; J.D.B. contributed new reagents/analytic tools; H.J. and L.C.B. analyzed data; and H.J., C.K., and S.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1510854112/-/DCSupplemental.

than in mature pollen. Because other sperm-specific genes (15, 16) exhibited similar expression ratios in microarray experiments, we predicted that in pollen *AHG3* might be restricted to sperm cells (i.e., not expressed in the vegetative cell).

AHG3 expression was assayed by quantitative RT-PCR (qRT-PCR) in unicellular microspores, mature pollen, and sperm cells. No *AHG3* transcripts were detected by qRT-PCR in unicellular microspores, and their levels in mature pollen were substantially lower than in sperm cells (Fig. 1*A*). Therefore, in mature pollen, *AHG3* transcripts accumulate mainly in sperm cells. To confirm the expression pattern of *AHG3* in pollen development, we generated transgenic plants with two constructs, a transcriptional fusion construct, Pro_{AHG3}*:NLS-3xGFP* (native promoter driving three copies of a GFP reporter gene with a nuclear localization sequence), and a translational GFP fusion construct, Pro_{AHG3}*:geAHG3-GFP* (the native promoter sequence driving the *AHG3* genomic coding sequence fused to a C-terminal GFP). We obtained 24 T1 lines with the promoter fusion; each mimicked the expression pattern previously reported (14, 15) in sporophytic tissues (Figs. S1 *A–F* and S2 *A* and *B*), indicating that the promoter in this construct was functional. During pollen development, NLS-3xGFP was not detected at unicellular or bicellular stages (Fig. 1 *B–E*) but was detected in mature pollen and pollen tubes in all 24 T1 lines only in the vegetative cell nucleus and not in sperm cell nuclei (Fig. 1 *F–I* and Fig. S3*A*). We obtained 40 T1 lines with the *geAHG3-GFP* protein fusion construct. To test if the promoter and AHG3-GFP in this construct were functional, *ahg3;* Pro_{AHG3}*:geAHG3-GFP* and *ahg3* control seeds were germinated for 5 d on Murashige and Skoog (MS) medium containing 0.3 uM ABA. The Pro_{AHG3}*:geAHG3-GFP* construct suppressed the ABA hypersensitive phenotype of *ahg3* seeds (Fig. S4), showing that the promoter fragment did confer correct expression of *AHG3* in sporophytic tissues. No GFP signal was detected in roots, leaves, or inflorescences under normal conditions (Fig. S1 *G–R*), but *geAGH3-GFP* expression was observed in nuclei in roots, leaves, and in some flower tissues upon ABA treatment, so AHG3 is an ABA-inducible protein localized in the nucleus (Fig. S2 *C–H*). In mature pollen and pollen tubes, *geAHG3-GFP* expression was detected in sperm cell nuclei (Fig. 1 *J–Q* and Fig. S3*B*). In addition to the strong signal in sperm cell nuclei, a weak geAHG3-GFP signal was detected in the vegetative cell nucleus of some pollen grains upon prolonged exposure (Fig. S3 *C* and *D*), but no signal was detected in sperm cell nuclei for the NLS-3xGFP fusion upon similar prolonged exposure (Fig. S3 *E* and *F*). To conclude, in pollen the site of *AHG3* transcription is different from AHG3 protein localization.

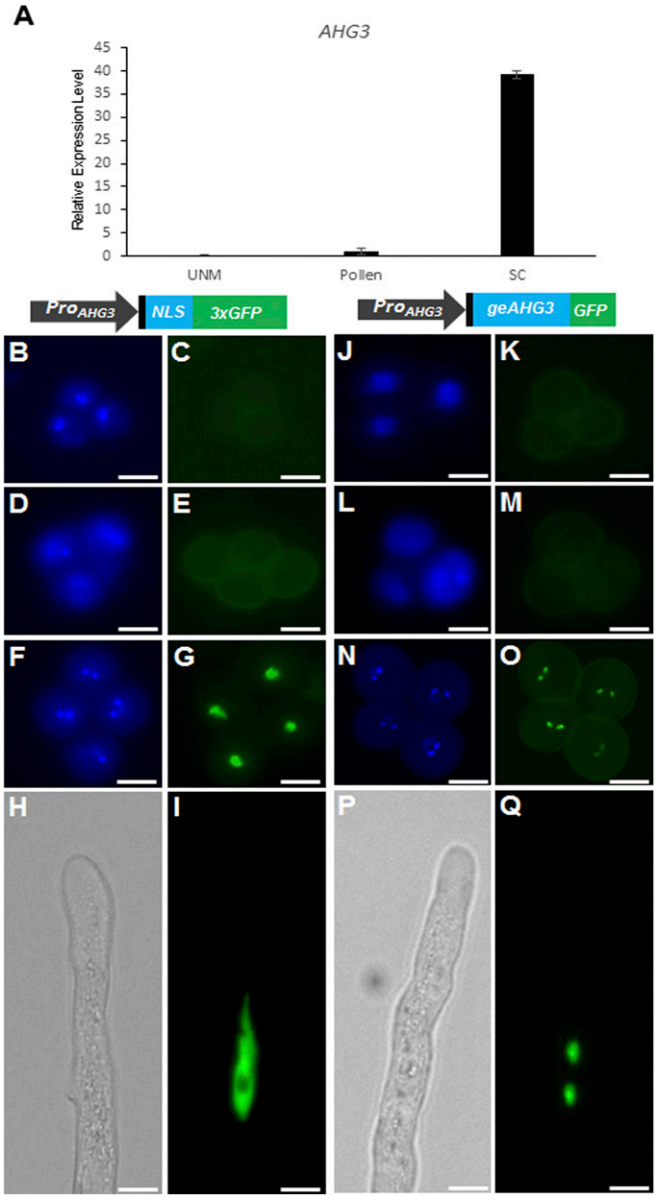

Fig. 1. *AHG3* transcriptional activity is different from AHG3 protein localization. (*A*) qRT-PCR analysis of *AHG3* transcript levels in unicellular microspores (UNM), mature pollen, and sperm cells (SC). (*B–Q*) Representative images showing the Pro_{AHG3}*:NLS-3xGFP* (*B–I*) or the Pro_{AHG3}*:geAHG3-GFP* expression patterns (*J–Q*) during pollen development. (*B*, *C*, *J*, and *K*) Unicellular microspores. (*D*, *E*, *L*, and *M*) Bicellular pollen. (*F*, *G*, N, and *O*) Mature pollen. (*H*, *I*, *P*, and *Q*) Pollen tubes. *B*, *D*, *F*, *J*, *L*, and *N* are DAPI images. *H* and *P* are brightfield images. *C*, *E*, *G*, *I*, *K*, *M*, *O*, and *Q* are GFP images. (Scale bars, 10 μm.)

A Combination of Sequences in the 5′ UTR and Coding Region Are Necessary for Transport to Sperm Cells. The different expression patterns of Pro_{AHG3}*:NLS-3xGFP* and Pro_{AHG3}*:geAHG3-GFP* in mature pollen suggested transport of transcripts or protein from the vegetative cell to sperm cells. To delimit the regulatory regions required for the transport, we generated two constructs corresponding to different regions of *AHG3* (Fig. 2*A*). In all 12 T1 lines with construct 1, which includes only the promoter and 5′ UTR sequence, the GFP signal was detected in the vegetative cell cytoplasm but not in the sperm cell cytoplasm (Fig. 2 *B* and *C* and Table 1). This localization was the same as that seen with Pro_{AHG3}*:NLS-3xGFP* (Fig. 1*G*), because the two constructs represent equivalent transcriptional fusions. In the 11 T1 lines with construct 2, which includes the sequence of construct 1 and the sequence encoding the N-terminal region of AHG3, the GFP signal was detected in the vegetative cell nucleus (Fig. 2*D*), in both the vegetative cell and sperm cell nuclei (Fig. S5*A*), or only in the two sperm cell nuclei (Fig. 2*E*). In more than half the lines all three patterns could be observed (Fig. S5*B*). We therefore concluded that transport of *AHG3* transcripts required the coding region, that the sequence encoding the N-terminal region in construct 2 was sufficient to assure partial transport, and that the sequence encoding the catalytic domain in construct 3 enhanced transport from the vegetative cell to the sperm cells.

To determine if the coding sequence of *AHG3* was sufficient for transport, we first used the *LAT52* promoter, which is expressed predominantly in the vegetative cell (10, 17), to drive expression of the *AHG3* genomic coding sequence (Pro_{LAT52}*:geAHG3-GFP*; construct 4 in Fig. 2*A*). The GFP signal in these lines was observed only in the vegetative cell nucleus (Fig. 2*G* and Table 1) but not in sperm cell nuclei. Thus, the coding DNA sequence (CDS) or genomic sequence alone was not sufficient to drive *AHG3* accumulation

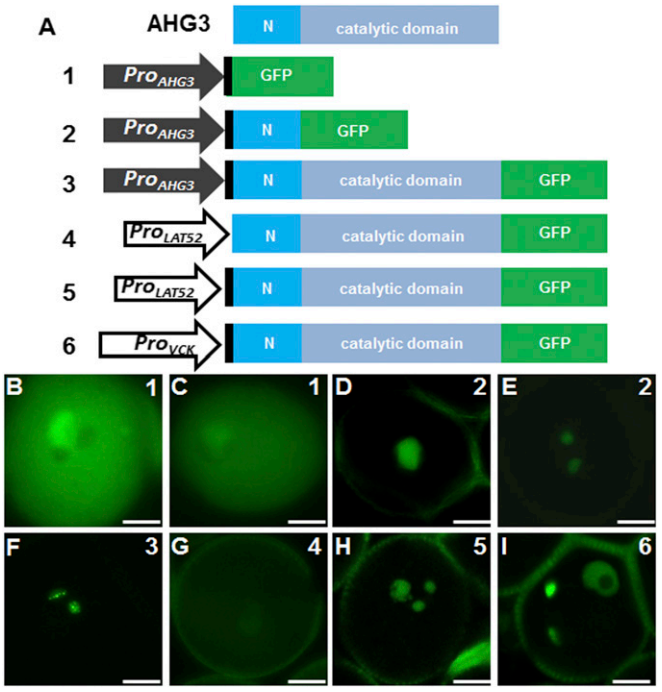

Fig. 2. A combination of 5′ UTR and coding sequences is necessary for the transport of *AHG3* transcripts to sperm cells. (*A*) AHG3 protein domains and diagram of constructs. The 5′ UTR is shown in black, the specific N terminal sequence is shown in blue, and the catalytic C-terminal domain is shown in gray. (*B–I*) Representative pollen grains expressing construct 1 (*B* and *C*), construct 2 (*D* and *E*), construct 3 (*F*), construct 4 (*G*), construct 5 (*H*), and construct 6 (*I*). (Scale bars, 5 μm.)

in sperm cells. Because the *AHG3* 5′ UTR was not included in construct 4 (Fig. 2*A*), we tested the requirement of the 5′ UTR for transport. We generated a *LAT52* promoter-driven construct that included both the *AHG3* 5′ UTR and the genomic coding sequence (Pro_{LAT52}*:5′UTR-geAHG3-GFP*, construct 5 in Fig. 2*A*). Unlike the constructs lacking the 5′ UTR, AHG3-GFP in this line was localized in both the vegetative cell nucleus and the sperm cell nuclei (Fig. 2*H*). However, because the *LAT52p* promoter was reported to drive weak expression at the unicellular microspore stage (10), the signal detected potentially could result from carry over from early stages of pollen development to sperm cells. To examine this possibility, we used the *VCK* promoter, a vegetative cell-specific promoter expressed first in late bicellular pollen (10), to drive expression of *AHG3* 5′ UTR plus the genomic coding sequence (Pro_{VCK}*:5UTR-geAHG3-GFP*, construct 6 in Fig. 2*A*). The pattern of GFP in this line was similar to that of lines with construct 5, i.e., AHG3-GFP was localized in both the vegetative cell nucleus and in the sperm cell nuclei. In mature pollen (from open flowers), about 20% of the pollen showed AHG3-GFP in both the vegetative cell nucleus and the sperm cell nuclei (Fig. S6 *A–G*), whereas at an earlier developmental stage (tricellular pollen from closed flowers) about half of the pollen had the AHG3-GFP signal in both the vegetative cell nucleus and the sperm cell nuclei (Fig. 2*M* and Fig. S6 *D–G*). The high proportion of pollen that had sperm cell localization in construct 9 lines strongly supports the hypothesis that sequences in the 5′ UTR are important for *AHG3* transcript transport. Together, these data suggest that *AHG3* transcripts and not AHG3 proteins are transported from the vegetative cell to sperm cells and that this transport requires sequences in both the 5′ UTR and the coding region of *AHG3*.

***AHG3* Transcripts Move from the Vegetative Cell to Sperm Cells.** To test the hypothesis that *AHG3* transcripts could move from the vegetative cell to sperm cells, we generated two additional constructs in which the *AHG3* promoter sequence drove expression of *AHG3* (referred to as "*AHG3-N*") comparable to construct 2 in Fig. 2*A* or a version with a mutated *AHG3* start codon (referred to as "ATG_{TtoA}*-AHG3-N*") (Fig. 3). As shown in Fig. 2*A*, 225 bp of the coding region of *AHG3-N* conferred partial ability for transcript movement from the vegetative cell to sperm cells. Because there is no other ATG codon in frame with the GFP in the N-terminal *AHG3* region, mutating the ATG codon of *AHG3-N* will result in the translation of only the *GFP*, even though both region *N* and *GFP* would be transcribed by the *AHG3* promoter. The expression pattern was observed in 10 T1 lines (each of the Pro_{AHG3}*:AHG3-N-GFP* and Pro_{AHG3}*:*ATG_{TtoA}*-AHG3-N-GFP* constructs). In 6 of 10 Pro_{AHG3}*:AHG3-N-GFP* lines, more than 50% of the pollen had only a sperm cell signal, whereas the remaining pollen had a signal either in the vegetative cell or in both the vegetative cell and the sperm cells (Fig. 3*G*). In three other lines more than 70% of the pollen had a signal only in the vegetative cell, whereas the remaining pollen had a signal in the vegetative cell and in the sperm cells. In the remaining line more than 90% of the pollen had a signal in both the vegetative cell and the sperm cells. In pollen with the Pro_{AHG3}*:*ATG_{TtoA}*-AHG3-N-GFP* construct, four lines had a cytoplasmic GFP signal in both the vegetative cell and the sperm cells, and four lines had a cytoplasmic GFP signal predominantly in sperm cells (Fig. 3 *H–N* and Fig. S7 *A* and *B*); only two lines showed exclusive vegetative cell expression (Fig. S7*C*). Although the percentage of pollen with complete sperm cell localization was less than that in pollen expressing Pro_{AHG3}*:AHG3-N-GFP*, the Pro_{AHG3}*:*ATG_{TtoA}*-AHG3-N-GFP* construct had the capacity to confer sperm cell localization of GFP (Fig. 3*N*). Together, these data strongly support our hypothesis that *AHG3* transcripts are transported from the vegetative cell to sperm cells, where they are locally translated.

To test this idea further, we generated two constructs in which the *AHG3* promoter sequence, including the 5′ UTR, drove expression of *GFP* in front of the *AHG3* genomic sequence (Fig. 4*A*). The first construct should produce GFP-AHG3, but the second construct should produce only GFP because of a stop codon introduced between the *GFP* and *AHG3* genomic sequences (*GFP-stop-AHG3*). In the majority of the Pro_{AHG3}*:GFP-AHG3* T1 lines (9 of 10), we observed a GFP signal in the vegetative cell nucleus or in both the vegetative cell nucleus and sperm cell nuclei (Fig. 4 *B* and *C–H*). Interestingly, in pollen that had GFP signals in vegetative cell and sperm cell nuclei, the intensity of the GFP signal in sperm cells was comparable to the sperm cell signal seen in pollen expressing Pro_{AHG3}*:geAHG3-GFP* (Fig. 2*F*), suggesting that transport of *GFP-AHG3* to sperm cell nuclei was not substantially impaired. However, the strong GFP signal in the vegetative cell nucleus implies that the *GFP-AHG3* mRNA is

Table 1. Pollen expression pattern of GFP lines

Name	No. of plants	VC	SC
Construct 1	12	+++	–
Construct 2	8	+	++
	3	+++	+
Construct 3	40	–	+++
Construct 4	20	+++	–
Construct 5	10	+++	++
Construct 6	10	+++	++

GFP signals in vegetative cells (VC) and sperm cells (SC) are noted as +, ++, and +++, representing weak, medium, and strong signals, respectively. For construct 2, two different expression patterns observed in transgenic lines are noted on separate lines.

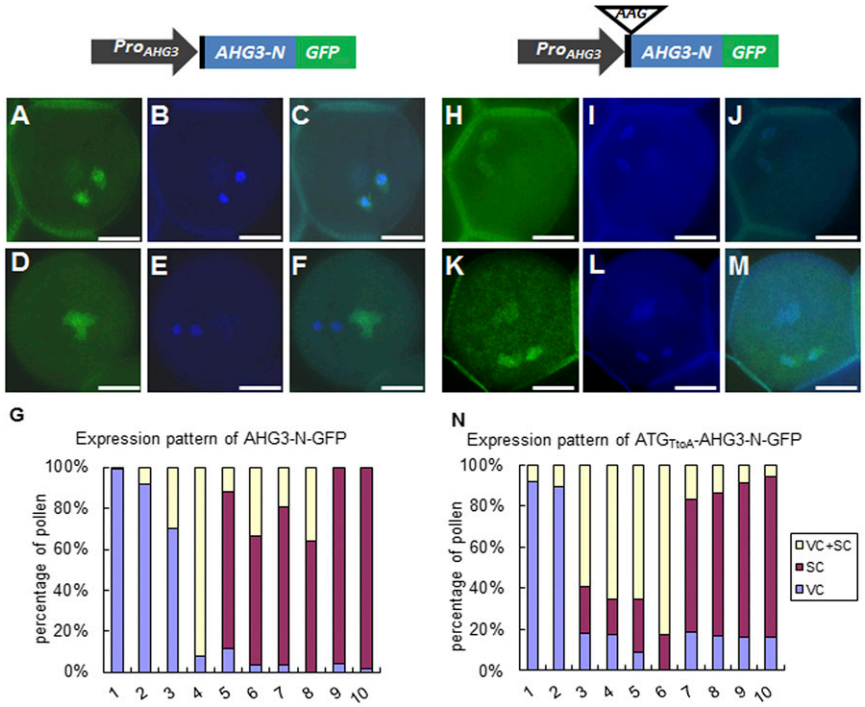

Fig. 3. GFP localization patterns of AHG3-N-GFP and ATG_{TtoA}-AHG3-N-GFP in mature pollen. The 5′ UTR is shown in black, GFP is shown in green, and AHG3 is shown in blue. The construct on the left contains the *AHG3* native promoter driving an *AHG3* genomic sequence with a C-terminally fused GFP (Pro_{AHG3}*:AGH3-N-GFP*); the construct on the right contains the same sequence except that the start codon in *AHG3* was mutated to AAG (Pro_{AHG3}*: ATG_{TtoA}-AGH3-N-GFP*). (*A–F*) Representative pollen grains expressing Pro_{AHG3}*:AHG3-N-GFP*. (*A* and *D*) GFP images. (*B* and *E*) DAPI images. (*C* and *F*) Merged GFP and DAPI images. (*G*) Percentage of localization patterns in pollen of 10 independent Pro_{AHG3}*:AGH3-N-GFP* transgenic lines. Each bar shows the distribution of localization patterns in one transgenic line. (*H–M*) Representative pollen grains expressing Pro_{AHG3}*: ATG_{TtoA}-AHG3-N-GFP*. (*H* and *K*) GFP images. (*I* and *L*) DAPI images. (*J* and *M*) Merged GFP and DAPI images. (*N*) Percentage of localization patterns in pollen of 10 Pro_{AHG3}*: ATG_{TtoA}-AHG3-N-GFP* transgenic lines. (Scale bars, 5 μm.) SC, sperm cells; VC, vegetative cells.

transcribed in the vegetative cell nucleus and translated in the cytoplasm; then the protein moves back to the vegetative cell nucleus. This signal clearly differs from the sperm-specific GFP signal in the lines expressing Pro_{AHG3}*:geAHG3-GFP* (Fig. 2*F*), suggesting that the N-terminal *GFP* fusion to *AHG3* disrupts a signal responsible for translational repression of *AHG3* in the vegetative cell. In 10 T1 lines with the *GFP-stop-AHG3* construct, the GFP signal was weak, even after prolonged exposure, suggesting that the stop codon triggered nonsense-mediated decay of the transcript (18). Nonetheless, there was some pollen with a weak GFP signal in both the vegetative cell cytoplasm and in sperm cells (Fig. 4 *I–K*), consistent with the idea that *AHG3* transcripts move from the vegetative cell to sperm cells.

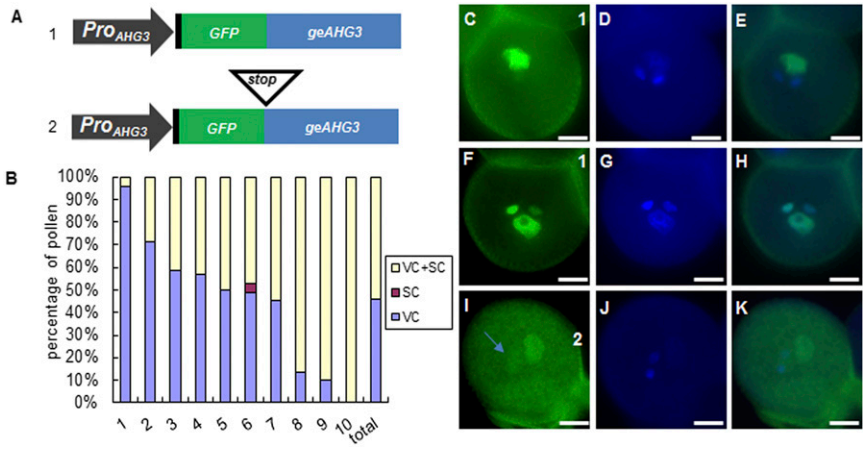

Fig. 4. Localization patterns of GFP-AHG3 and GFP-Stop-AHG3. (*A*) Diagrams of constructs. The 5′ UTR is shown in black, GFP is shown in green, and *AHG3* is shown in blue. (*B*) Percentage of localization patterns in pollen expressing Pro_{AHG3}*:GFP-geAHG3*. The data represent pollen from 10 independent T1 lines, and the total bar shows the average from 10 T1 plants. SC, sperm cells; VC, vegetative cells. (*C–K*) Representative images of pollen expressing Pro_{AHG3}*:GFP-geAHG3* (*C–H*) or Pro_{AHG3}*:GFP-Stop-geAHG3* (*I–K*). *C*, *F*, and *I* are GFP images. *D*, *G*, and *J* are DAPI images. *E*, *H*, and *K* are merged GFP/DAPI images. The arrow in *I* marks GFP in sperm cell nuclei. (Scale bars, 5 μm.)

Discussion

Here we show that *AHG3* transcripts move from the vegetative cell to sperm cells. Although more than 20 mobile endogenous mRNAs have been reported in other tissues in plants (reviewed in ref. 11), intercellular mRNA communication in pollen was not previously documented. We provide multiple lines of evidence to support this claim. (*i*) The AHG3 protein localized specifically in sperm cells, whereas transcription of *AHG3* occurred in the vegetative cell. (*ii*) Pollen of Pro_{AHG3}*:* ATG_{TtoA}*-AHG3N-GFP* lines (a nontranslatable fusion of a 225-bp portion of *AHG3* to *GFP* under the control of Pro_{AHG3}) also showed predominant sperm cell localization. (*iii*) Pollen of Pro_{LAT52}*:geAHG3-GFP* lines (only the *AHG3* coding sequence under the control of Pro_{LAT52}) did not confer sperm cell localization, but pollen of the Pro_{LAT52}*:5′UTR-geAHG3-GFP* and Pro_{VCK}*:5′UTR-geAHG3-GFP* lines (in which the 5′ UTR and *AHG3* coding sequence are under the control of Pro_{LAT52} or Pro_{VCK}, respectively) had obvious sperm cell localization, showing that the *AHG3* coding region alone is not sufficient for mRNA movement but also requires the 5′ UTR sequence. (*iv*) Both the microarray dataset from sperm cells (13) and our qRT-PCR results (Fig. 1*A*) indicated substantially higher levels of *AHG3* mRNA in sperm cells than in pollen. Together, our data strongly support the idea that the presence of the AHG3 protein in sperm cells is dependent mainly on RNA movement from the vegetative cell to sperm cells and that this mRNA transport depends on both the *AHG3* 5′ UTR and coding sequence. There is precedent for the requirement of UTRs and coding sequences for RNA movement and subcellular localization. For example, in *Saccharomyces cerevisiae*, a motif in the 3′ UTR together with three motifs in the coding region are important for movement of *ASH1* mRNA (19). Similarly, in *Arabidopsis*, motifs in both the 3′ UTR and coding region are important for *GAI* mRNA movement (20).

RNA localization is a mechanism for regulating gene expression posttranscriptionally. In animals, localized RNAs are important determinants of cell fate in eggs and embryos, and such localization allows spatially restricted synthesis of specific proteins in distinct regions of the cytoplasm (21). During animal spermiogenesis, mRNA localization and translation are regulated in a stage-specific manner (22–24). During spermiogenesis, DNA-binding histones are replaced by protamines, resulting in chromatin condensation and cessation of transcription in elongating spermatids (25, 26). Hence, in haploid spermatids, transcription and translation are temporally uncoupled to ensure protein synthesis in transcriptionally silent germ cells. The storage of translationally repressed transcripts is a common phenomenon in haploid spermatids, resulting in temporal differences in the occurrence of mRNAs and their corresponding proteins (27, 28).

In plants, as in animals, the chromatin of sperm cells is highly condensed and contains a male gamete-specific histone H3 that might serve a function similar to that of protamines in animals (29). However, transcriptome studies of isolated sperm cells showed that sperm cells contain thousands of transcripts, consistent with transcriptional activity (13, 30). This idea is supported by the identification of several sperm cell-specific genes, such as *GEX1* and *DUO1* (31, 32). Nevertheless, the extent of active transcription in plant sperm cells remains to be investigated, because some transcripts identified in the sperm cell transcriptome might have been transported from the vegetative cell during pollen development. By analyzing the incorporation of 5-BrUra into RNA in Hyacinthus pollen, sperm cell nuclei were shown to be transcriptionally active, although at a markedly lower level than in the vegetative cell nucleus (33). *AHG3* might represent a class of genes that are transcribed but translationally repressed in the vegetative cell in tricellular pollen and whose mRNAs are transported to sperm cells where they are locally translated. This view is strongly supported by our finding that an N-terminal fusion of *GFP* to *AHG3* caused the accumulation of GFP-AHG3 in the vegetative cell nucleus, possibly by disrupting elements between the 5′ UTR and translational start codon required for the translational repression of *AHG3* in the vegetative cell. The mechanisms responsible for translational repression are currently unknown, as are the intercellular routes promoting transport within the male germ unit in pollen. However, *AHG3* is most likely not an exceptional case, because it has been shown that ribonucleoprotein particles in pollen contain translationally silent mRNAs (34). These ribonucleoprotein complexes have been interpreted to serve as long-term storage of mRNAs that are transported to the pollen tube tip, along with the translational machinery, during pollen tube growth. Analogously, a subset of these mRNAs might move to and be translated in sperm cells. The mechanisms for mRNA and small RNA movement in sporophytic tissues reported so far differ (11, 12); therefore, we cannot make any assumptions about mechanisms for the transport of small RNAs or mRNAs between the vegetative cell and sperm cells; these mechanisms must be addressed using different experimental strategies. In conclusion, our findings document that the vegetative cell provides protein-encoding transcripts to sperm cells.

Materials and Methods

Seed and Plant Growth Conditions. Seeds of the *ahg3* mutant (CS851888) were obtained from the *Arabidopsis* Biological Resource Center. Plants were grown in a greenhouse in a 4:1:1 mix of Fafard 4P:perlite:vermiculite under an 18-h light/6-h dark cycle at 21 °C. For ABA treatments of the Pro_{AHG3}*:NLS-3xGFP* and Pro_{AHG3}*:geAHG3-GFP* lines, seeds were germinated on MS medium for 7 d and then were transferred to medium containing 100 μM ABA for 2 d. For complementation tests of the *ahg3* mutant, *ahg3;*Pro_{AHG3}*:geAHG3-GFP* and *ahg3* control seeds were germinated on MS medium containing 0.3 μM ABA for 5 d (35). For ABA treatment of inflorescences, inflorescences were cultured as described in ref. 36. Briefly, inflorescences were cut from the plants, and all open flowers were removed. The inflorescences were inserted immediately through a hole in the lid of a microtiter plate into MS medium containing 100 μM ABA. The plates were incubated in a 22 °C growth chamber for 4 d.

Plasmid Constructions. *AHG3* was amplified from Col-0 genomic DNA and cloned into pENTR-D/TOPO, then transferred into a plant expression destination vector modified from pB7FWBG2 (37) (35S promoter removed). The *AHG3* CDS was amplified from pollen cDNA with the primers specified in Table S1 and cloned into pENTR-D/TOPO (Invitrogen), then transferred into a plant expression destination vector modified from pZY06 (38). The resulting plasmid was introduced into *qrt1* plants (39) by Agro-infiltration (40). For the *AHG3* promoter, a 1,472-bp fragment upstream of the ATG codon was subcloned into pENTR-D/TOPO and then transferred into the plant expression vector pGII-NLS3XGFP (15). To generate *ProLAT52:5UTR-geAHG3-GFP* and *ProVCK:5UTR-geAHG3-GFP*, we replaced the 35S promoter in pB7FWG2.0 with the *VCK* promoter (identical to that used in reference 10) and then introduced the 5′ UTR and *AHG3* genomic sequence into this vector and the pZY06 vector. To generate *ProAHG3:GFP-AHG3* and *ProAHG3:GFP-Stop-AHG3*, we replaced the 35S promoter in pB7FWG2.0 with the *AHG3* promoter and then introduced a wild-type *AHG3* genomic sequence or a mutated *AHG3* genomic sequence without ATG into this vector. To generate *ProAHG3:AGH3-N-GFP* and Pro_{AHG3}*:-AHG3-N-GFP*, we introduced a wild-type region N or a mutated (without ATG) region N into the vector.

Microscopic Analysis of Transgenic Lines. Pollen from closed buds and open flowers of transgenic T1 plants were collected as described in ref. 41. Images were acquired with an Axiovert microscope (Zeiss) using the epifluorescence channel, an AxioCamRM camera, and AxioVision 4.3 software, and a Zeiss confocal microscope LSM 780. Images were processed using Adobe Photoshop 7.

Real-Time PCR. Purified FACS samples from unicellular microspores, pollen, and sperm cells were isolated as described in ref. 42. Total RNA was extracted using the Qiagen RNeasy Micro Kit according to the manufacturer's instructions. Expression levels were analyzed using qRT-PCR, using 10 ng of amplified cDNA resulting from aliquots of cRNA synthesized following the Ambion WT Expression kit protocol for hybridization with Affymetrix GeneChip Whole Transcript Expression Arrays. All real-time PCR reactions

were performed using the ABI7900 Sequence Detection System (Applied Biosystems), and the amplifications were done using the SYBR Green FastMix ROX (Quanta BioSciences). The relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (43); fold changes in gene expression were normalized to the internal control (*HTA10*) and relative to the calibrator sample (pollen). There were three biological replicates in three independent experiments.

ACKNOWLEDGMENTS. We thank Sarah Chan and Wonsang Tahk, participants in the University of California, Berkeley undergraduate research apprentice program, for technical assistance and Binglian Zheng, Guang Wu, and Peng Qin for useful suggestions and comments on the manuscript. This work was supported by US Department of Agriculture-Agricultural Research Service Current Research Information System Grant 5335–21000–030–00D (to S.M.), by a European Research Council Starting Independent Researcher grant (to C.K.), and by Fundação Ciência e Tecnologia Grant EXPL/BIA-PLA/0244/2013 (to L.C.B.).

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