Report

The Peroxin Loss-of-Function Mutation *abstinence by mutual consent* Disrupts Male-Female Gametophyte Recognition

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Summary

In eukaryotes, fertilization relies on complex and specialized mechanisms that achieve the precise delivery of the male gamete to the female gamete and their subsequent union [1–4]. In flowering plants, the haploid male gametophyte or pollen tube (PT) [5] carries two nonmotile sperm cells to the female gametophyte (FG) or embryo sac [6] during a long assisted journey through the maternal tissues [7–10]. In *Arabidopsis*, typically one PT reaches one of the two synergids of the FG (Figure 1A), where it terminates its growth and delivers the sperm cells, a poorly understood process called pollen-tube reception. Here, we report the isolation and characterization of the *Arabidopsis* mutant *abstinence by mutual consent* (*amc*). Interestingly, pollen-tube reception is impaired only when an *amc* pollen tube reaches an *amc* female gametophyte, resulting in pollen-tube overgrowth and completely preventing sperm discharge and the development of homozygous mutants. Moreover, we show that *AMC* is strongly and transiently expressed in both male and female gametophytes during fertilization and that AMC functions in gametophytes as a peroxin essential for protein import into peroxisomes. These findings show that peroxisomes play an unexpected key role in gametophyte recognition and implicate a diffusible signal emanating from either gametophyte that is required for pollen-tube discharge.

Results and Discussion

To date, the molecular and genetic mechanisms of pollen-tube reception are poorly understood, and only the *Arabidopsis feronia/sirene* mutations have been reported to specifically disrupt this complex process [11, 12]. In the *feronia/sirene* female gametophytic mutants, pollen tubes (PTs) reach the micropyle but are unable to stop their growth and are unable to burst, demonstrating that the female gametophyte (FG) participates in the

control of PT reception [11, 12]. With the recent characterization of FER/SIR as a synergid-expressed, plasma-membrane-localized receptor-like kinase [13], one possible model for pollen-tube reception emerges: When the PT reaches the synergids, a ligand from the PT triggers upon binding to the FER extracellular domain a signaling cascade, enabling the female gametophyte to prepare itself for fertilization [13, 14]. In return, the FG would signal back to the pollen tube to stop growing and discharge its sperm cells [13, 14]. Here, we have identified and characterized a transfer DNA (T-DNA) insertional *Arabidopsis* mutant that exhibits defective pollen-tube reception only when a mutant pollen tube interacts with a mutant embryo sac, resulting in pollen-tube overgrowth and the absence of homozygous individuals. Therefore, we named this self-sterile mutant *amc*/+ for *abstinence by mutual consent*.

amc Mutant Does Not Produce Homozygous Individuals

After screening more than 500 progenies from self-pollinated *amc*/+ T2 plants, we were unable to identify plants homozygous for the T-DNA insertion in the *AMC* gene (Table 1). Unlike immature siliques of wild-type (WT) plants filled to 94% with green seeds, self-pollinated heterozygous *amc*/+ siliques were only filled to 76% with green seeds and contained also 24% of white shriveled ovules randomly located within the siliques (Table 1; Figure 1B). Within *amc*/+ progeny, the segregation of the *amc* allele (Table 1) or the Kanamycin marker from the T-DNA (Table S1 available online) resulted in an approximately 2:1 ratio instead of the expected 3:1. The absence of homozygous plants, the seed-set silique phenotype and the distorted segregation ratio were still occurring after four successive backcrosses, and all of the Kanamycin-resistant plants (n > 300) exhibited the incomplete seed-set phenotype.

We performed reciprocal crosses to the wild-type to determine the transmission efficiency (TE) of the *amc* allele. Although the transmission by the female gametophyte was not significantly affected (83%; Table 1; $\chi^2 = 0.49$, $0.5 > p > 0.4$), the TE by the male gametophyte was moderately but significantly reduced (51%; Table 1; $\chi^2 = 10.45$, $p < 0.01$). Furthermore, siliques resulting from either reciprocal cross had a wild-type-like full seed set (Table 1), indicating that the reduced male TE could not alone account for the incomplete silique seed set observed during *amc*/+ self-pollination. Because the *amc* mutation is fully penetrant and affects neither pollen germination nor PT growth in vitro (see below and Supplemental Data, Figure S7), one possible explanation for the reduced male TE is that *amc* mutant pollen tubes are less efficient than wild-type PTs at targeting ovules. More importantly, these findings indicate that the observed ovule abortion within *amc*/+ siliques is caused by an embryonic defect or a synergistic defect from both male and female gametophytes. Considering the reduced male TE, within the progeny

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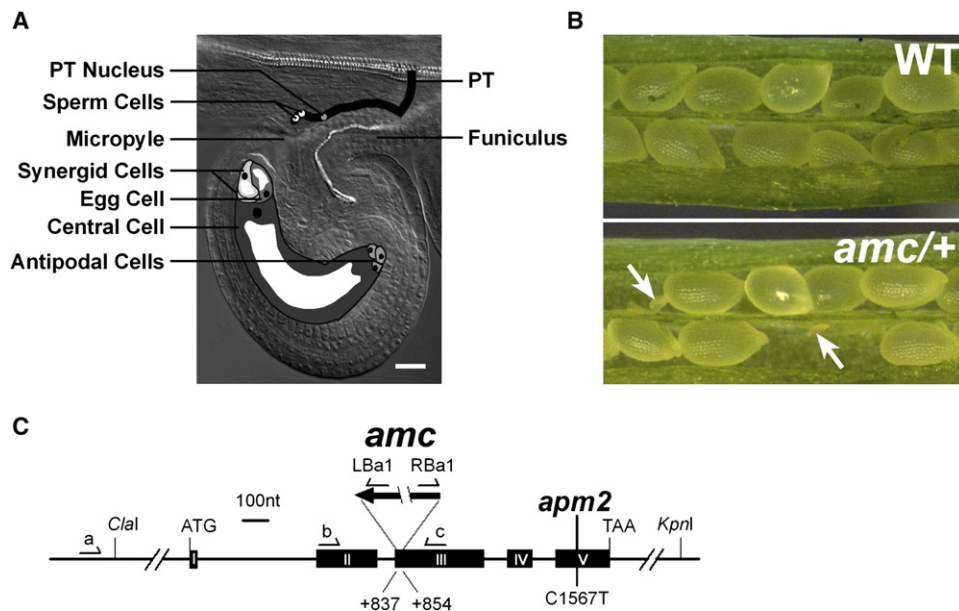
Figure 1. Siliques of *amc*/+ Mutant Lack Homozygous Individuals

(A) Schematic drawing of male and female gametophytes during fertilization in *Arabidopsis thaliana*. The pollen tube (PT) enters the ovule through the micropyle, an opening leading to the seven-celled haploid female gametophyte and reaches the receptive synergid cell in which it will discharge its sperm cells, a process called pollen-tube reception. During double fertilization, one sperm cell fuses with the egg cell, which will give rise to the zygote, whereas the second sperm will fertilize the central cell to induce endosperm formation. The scale bar represents 20 μm.

(B) In contrast to wild-type siliques (top), siliques of self-pollinated *amc*/+ plants frequently contained degenerating ovules (bottom, white arrows).

(C) Exon and intron organization of the *APM2/AMC* locus (At3g07560) and position of the T-DNA in the *amc*/+ mutant. Positions of the primers used to genotype the *amc*/+ mutant and position of the point mutation in the weak *apm2* allele [15] are indicated.

of self-pollinated *amc*/+ plants, homozygous *amc/amc* individuals should be observed at a frequency of 15.3% (Table S2; $0.337 \times 0.453 = 0.153$) instead of the expected 25% ($0.5 \times 0.5 = 0.25$). However, such homozygous plants were never identified (n = 524, Table 1).

AMC Encodes an Atypical Peroxin

The *amc*/+ mutant carries a T-DNA insertion in exon III of the At3g07560 gene that triggered a 16 bp deletion, suggesting that *amc* is a frame-shifted disruption allele (Figure 1C). Introduction of a wild-type copy of At3g07560 rescued the seed-set phenotype in 13 out of 16 *amc*/+ T1 lines. The absence of homozygotes and the distorted segregation ratio observed for *amc*/+ plants and their progeny were also complemented (see Supplemental Data), demonstrating that At3g07560 corresponds to the *AMC* gene. *AMC* encodes a protein of 304 amino acids and is a single gene in *Arabidopsis thaliana*.

Recently, Mano and colleagues reported that the *aberrant peroxisome morphology 2* (*apm2*) mutant,

which carries a point mutation in the C-terminal region of AMC (Figure 1C), from here on named APM2/AMC, exhibits weak peroxin-deficient phenotypes [15]. They also showed that APM2/AMC is targeted to peroxisomes and exhibits limited similarity to the SH3-domain-containing peroxin PEX13 from various organisms (see Supplemental Data, Figure S1B) [15]. Most peroxisomal proteins use either peroxisome targeted signal 1 (PTS1) or PTS2 to enter peroxisomes [16, 17], and PEX13 has been shown to function in both PTS1- and PTS2-dependent import pathways [18, 19]. However, our detailed APM2/AMC sequence analyses indicated that APM2/AMC does not have an SH3 domain and might not be a direct ortholog of PEX13 (see Supplemental Data, Figure S1).

APM2/AMC Is Essential for Pollen-Tube Discharge

To investigate the cause of the incomplete seed set of selfed *amc*/+ siliques, we analyzed developing seeds in *amc*/+ siliques by using Nomarski optics but could

Table 1. Segregation Analysis of the *amc* Mutation by PCR-Based Genotyping

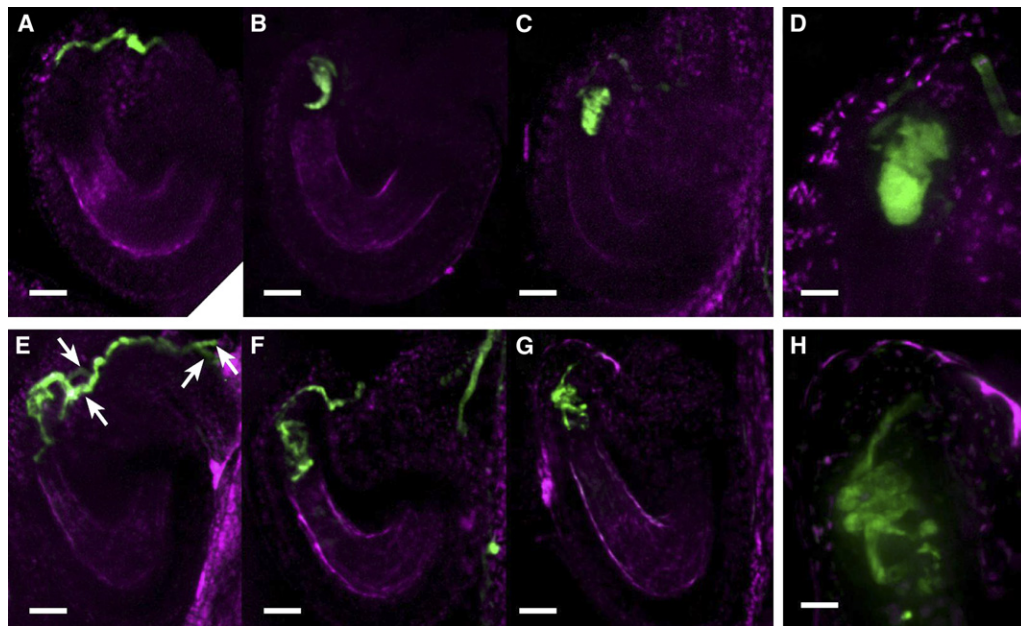| Female × Male	+/+	*amc*/+	*amc/amc*	Percent *amc*/+	Percent TE	Percent of Nonaborted Ovules in F1 Siliques
amc/+ × *amc*/+	218	306	0	58.4	nd	76 (n = 891)
amc/+ × +/+	29	24	0	45.3	82.8	94 (n = 605)
+/+ × *amc*/+	65	33	0	33.7	50.8	93 (n = 923)
+/+ × +/+	nd	nd	nd	nd	nd	94 (n = 712)

"nd" indicates not determined.

Figure 2. The *amc* Mutation Disrupts Pollen-Tube Growth Arrest and Discharge

Examination by confocal microscopy of YFP-expressing pollen 19–24 hr after manual pollination during wild-type (A–D) and *amc* mutant (E–H) pollen-ovule interactions. Fluorescence from the YFP expressed in pollen tubes is shown in green, and the autofluorescence from the ovule tissues is shown in magenta. Scales bars represent 23 μm for (A)–(C), (E)–(G), and 7 μm for (D) and (H).

(A) A wild-type pollen tube reaching the micropylar side of the synergid cells.

(B and C) Two examples of wild-type pollen-tube discharges. At the extremity of the PT, the fluorescence assumes the shape of the receptive synergid cell in which the pollen tube has released its content.

(D) Close-up of (C).

(E–G) Three examples of continued growth of presumably an *amc* pollen tube reaching an *amc* ovule. The mutant pollen tube, although reaching the micropylar part of the synergid cells, does not discharge. It keeps growing by coiling and branching (F and G), and the pollen-tube tips can be spotted past the synergid cells of the mutant ovule (E and F). Among these mutant interactions, two pollen tubes can be frequently observed within a presumed *amc* ovule ([E], white arrows).

(H) Close-up image of the coiled pollen tube shown in (G). Note how the pollen tube coils and branches, making the fluorescence outlines rougher than in (D).

not observe any obvious fertilization signs in the senescing ovules (see Supplemental Data, Figure S2A). Moreover, within *amc*/+ siliques, the small senescing ovules were targeted by PTs as frequently as the normal young seeds (see Supplemental Data, Figure S2B) suggesting that the incomplete seed set observed for the *amc*/+ siliques is the result of ovules normally targeted by PTs that remain unfertilized. Therefore, it is very likely that the *amc* mutation triggers defects in fertilization.

So that the effect of the *amc* mutation on the interactions between gametophytes could be visualized, transgenic wild-type and *amc*/+ plants expressing the yellow fluorescent protein (YFP) reporter driven by the pollen-specific *ACA9* promoter were generated [20]. When wild-type YFP-expressing pollen was deposited on wild-type pistils, two distinct interactions were observed 19–24 hr after manual pollination. In approximately 64% of the wild-type events, one PT tip reached the vicinity of the micropylar side of the synergids (n = 216 out of 336; Figure 2A). This could be interpreted as the PT having reached the synergids and not having discharged yet or the transient discharge having already occurred. In the remaining interactions, as previously described [21], a classical transient discharge of one fluorescent pollen tube content within the receptive

synergid could be observed (n = 120 out of 336; Figures 2B–2D). In manual crosses between pollen and pistils from *amc*/+ plants, we also witnessed PTs at the micropylar side of synergids (n = 275 out of 468), as well as classical discharges (n = 130 out of 468). Surprisingly, in the remaining 13.5% of the interactions (n = 63 out of 468), the pollen tube normally reached the synergids, but then, instead of terminating its growth and bursting, it invaded the micropylar part of the ovule by growing continuously and forming coils and/or branching (Figures 2E–2H). Moreover, during these mutant interactions, synergid cell degeneration appeared to be delayed, although PTs and synergids were in close contact (see Supplemental Data, Figure S3) [22]. Such severe PT overgrowth was never observed during reciprocal crosses of *amc*/+ with wild-type (n > 300 for both cross directions), consistent with the wild-type-like full seed set of the siliques resulting from these crosses (Table 1).

Among the interactions between *amc*/+ pollen and *amc*/+ pistils, the invading-pollen-tube phenotype was observed in 13.5% of the analyzed events, which was not significantly different from the 15.3% (χ^2 = 1.22, 0.3 > p > 0.2) expected for the interaction between an *amc* mutant pollen and an *amc* mutant ovule (see above and Table S2). Thus, our data provide strong evidence that during the interaction between an *amc* mutant

pollen tube and an *amc* mutant embryo sac, the PT keeps growing and does not deliver its sperm cells, therefore preventing fertilization and the development of an *amc/amc* homozygous individual.

Although wild-type ovules typically receive only one pollen tube [9], an *amc* ovule receiving an *amc* PT appears to continue to attract other pollen tubes because we frequently observed more than one PT within the FGs with the invading-pollen-mutant phenotype (n = 28 out of 63; Figure 2E, arrows).

Interestingly, the *fer/sir* mutants exhibit very similar invading-pollen-tube phenotypes to the ones observed for *amc/+*, although in the case of the *feronia/sirene* mutants, they are only attributable to the mutant FGs [11, 12]. Therefore, our data provide genetic evidence that pollen tubes are not just a passive sperm carrier controlled by the FG during pollen-tube reception. Strikingly, the failure of pollen-tube reception in *feronia/sirene* FGs or between *amc* PTs and *amc* FGs appeared to have the same immediate consequences: the receptive synergid does not degenerate upon arrival of the pollen tube, and the female gametophyte continues to attract other PTs (Figure 2E and Figure S3C) [11, 12].

Because *feronia* female gametophytes are unable to correctly receive wild-type PTs, one could argue that the PT-overgrowth phenotype is a consequence of mutant FGs structurally or physiologically unfit to receive pollen tubes [11, 12]. However, the new finding that *amc* FGs are fully competent to receive wild-type PTs (Table 1) added to the observations that *feronia* and *amc* female gametophytes correctly expressed synergid-specific markers and did not reveal any structural defect (see Supplemental Data, Figure S3) [11] rules out this possibility. An emerging alternative model for the pollen-tube overgrowth observed in these mutants is that a FER receptor kinase-dependent recognition-communication system between male and female gametophytes that is necessary for proper pollen-tube reception is disrupted [13, 14]. Analysis of the seed set of self-pollinated *fer/+ amc/+* double-mutant siliques suggests that the FER and APM2/AMC pathways are at least partially independent (see Supplemental Data and Table S3). The *amc* mutation might disrupt either the FER-dependent signaling pathway in the FG or the subsequent feedback signaling pathway in the PT that leads to sperm discharge. Alternatively, redundant signals or molecules independent from the FER-dependent pathway and originating from either gametophyte could be required to create a unique niche suitable for sperm discharge.

APM2/AMC Is Expressed in Both Male and Female Gametophytes during Fertilization

Consistent with publicly available ATH1 microarray data from various organs [23] and pollen transcriptome studies [24, 25] (Figure S4A), *APM2/AMC* expression was detected by semiquantitative and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analyses in every organ tested but was particularly more abundant in pollen (Figure 3A). In transgenic lines expressing the beta-glucuronidase gene (*GUS*) driven by a 1.4 Kb long *APM2/AMC* promoter, moderate GUS activity was present in a wide range of vegetative tissues (see Supplemental Data, Figure S4), and again,
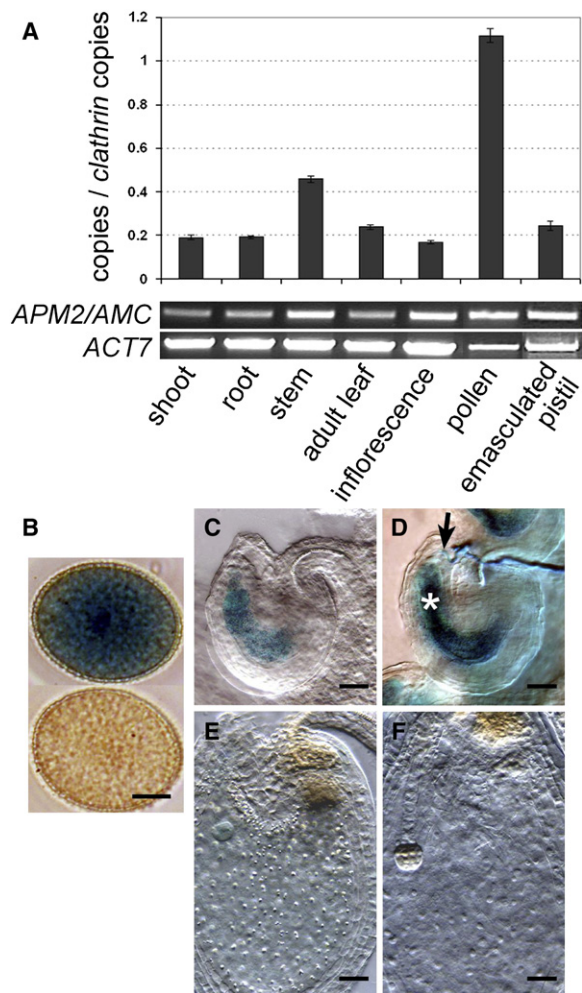

Figure 3. *APM2/AMC* Expression Pattern Supports Its Role during Fertilization

(A) Semiquantitative RT-PCR (bottom, 31 cycles) and quantitative real-time PCR (top) analyses of *APM2/AMC* expression in different organs or tissues. *ACTIN7* (At5g09810) was used as a control for RT-PCR analysis. For quantitative real-time PCR experiments, *Clathrin* (At4g24550) was used as an internal control, and each data point indicates the average of three independent experiments ± the standard error of the mean (SEM). In line with the microarray data (Figure S4A), these analyses indicated that *APM2/AMC* is preferentially expressed in mature pollen grains.

(B–F) Histochemical localization of *GUS* reporter gene expression driven by the *APM2/AMC* promoter in pollen grains (B) and during fertilization (C–F). (B) shows 1 hr GUS-stained *pAMC-GUS* pollen (top) and untransformed wild-type pollen (bottom) grains. The scale bar represents 5 μm. (C)–(F) show *GUS* expression in a mature female gametophyte before (C), during (D), 24 hr after (E), and 48 hr after (F) fertilization (12 hr GUS staining). During fertilization (D), both male (arrow) and female (asterisk) gametophytes are strongly stained. Note how strongly the GUS activity dropped after fertilization (E and F). Scale bars represent 28 μm for (C)–(F). See also Supplemental Data and Figure S4F.

the strongest GUS signal was observed in the mature pollen grain (Figure 3B). Interestingly, in the unfertilized mature female gametophytes, GUS activity was induced by pollen deposition on the pistils (Figure 3C, see also Supplemental Data and Figure S4F for whole stained-pistil series during pollination). During fertilization, both female (asterisk) and male (arrow) gametophytes

were intensely stained, in clear contrast to the surrounding diploid tissues (Figure 3D). However, after fertilization, *APM2/AMC* promoter activity decreased dramatically as the young embryo developed (Figures 3E–3F). Together, our data indicate that *APM2/AMC* is strongly expressed in both male and female gametophytes during fertilization, consistent with an important fertilization function for APM2/AMC in both gametophytes.

The *amc* Mutation Completely Disrupts PTS1-Dependent Protein Import into Pollen Peroxisomes
First, we confirmed the peroxisomal subcellular localization of APM2/AMC [15] in onion epidermal cells (see Supplemental Data, Figure S5). Moreover *APM2/AMC* antisense lines exhibited phenotypes in vegetative tissues that are characteristic of peroxin mutants deficient in photorespiration, a peroxisomal process requiring PTS1-dependent import (see Supplemental Data, Figure S6) [26–29]. Consistently, the protein import into peroxisomes via the PTS1-dependent pathway was indeed partially defective in the *APM2/AMC* antisense lines (see Supplemental Data, Figure S6), as reported for the weak *apm2* mutant [15]. Finally, we directly analyzed the effect of the *amc* null mutation on PTS1-dependent protein import into peroxisomes by transforming WT and *amc/+* plants (in a *quartet* mutant background) [30] with the pollen peroxisome targeting pLAT52-CFP-PTS1 construct [31]. In WT plants heterozygous for the pLAT52-CFP-PTS1 fusion, all of the fluorescent pollen grains exhibited fluorescence with a peroxisome-like punctuated pattern (Figure 4A, arrows; n > 200). In clear contrast, pollen grains from *amc/+* tetrads exhibited fluorescence exclusively either in peroxisomes (Figure 4B, arrow; n = 117 out of 242) or in the cytosol (Figure 4B, arrowhead; n = 125 out of 242) in the 1:1 ratio expected for the segregation of the *amc* mutation ($\chi^2 = 0.26$, $0.7 > p > 0.5$; see also Figure S7). Therefore, our results provide strong evidence that APM2/AMC functions as a peroxin in reproductive tissues and more importantly that APM2/AMC is essential for the PTS1-dependent import pathway. Although the precise relation of APM2/AMC to other peroxins requires further investigation, analysis of the strong *amc* allele definitely establishes APM2/AMC as a core component of the plant peroxisomal matrix protein import machinery.

The present detailed analysis of the complete loss-of-function *amc* allele also reveals for the first time that functional peroxisomes must be present in either the male or the female gametophyte for pollen-tube reception to take place. It is therefore conceivable that the mislocalization of a protein normally targeted to the peroxisomes can affect pollen-tube reception. Peroxisomes in plants are known to maintain a cellular redox balance and are a source of a large range of signaling molecules, such as jasmonic acid, salicylic acid, indole acetic acid, reactive oxygen species (ROS), and nitric oxide (NO) [32, 33]. In animals, ROS and NO are known to play key roles in fertilization-related processes, such as capacitation, the acrosome reaction, oocyte activation, ovulation, and fertilization itself [34–37]. In plants, little is known about the functions of these signaling molecules during fertilization. Recently, peroxisomes have been shown to be the source of NO
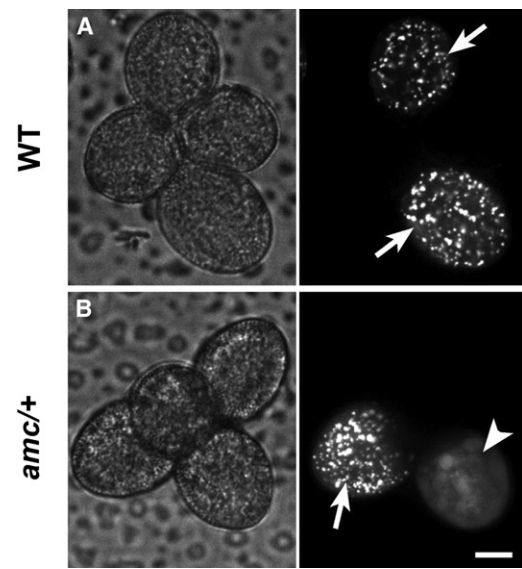

Figure 4. PTS1-Dependent Protein Import into Peroxisomes Is Completely Impaired in *amc* Mutant Pollen

Examination by confocal microscopy of pollen tetrads of wild-type (A) and *amc/+* (B) plants heterozygous for the peroxisomal pLAT52-CFP-PTS1 marker. Bright light is shown in the left panels, and CFP filter is used in the right panels. The scale bar represents 10 μm.
(A) In wild-type tetrads, all of the fluorescent pollen grains (half of the total grains) exhibited a fluorescence with a peroxisome-like punctuated pattern (white arrows), indicative of a normal PTS1-dependent import into peroxisomes.
(B) In *amc/+* tetrads, in a 1:1 expected ratio for segregation of the fully penetrant *amc* mutation, fluorescent pollen grains exhibited fluorescence either in a punctuated pattern as in wild-type (arrow) or exclusively in the cytosol (arrowhead). In this latter case, note how peroxisomes can no longer be discerned, indicating complete impairment of PTS1-dependent import into peroxisomes (see also Figure S7 for pollen tubes growing in vitro).

production in pollen tubes, and NO was able to reorient pollen-tube growth in vitro [31]. One possible model for our findings is that a signal or molecule originating from gametophyte peroxisomes—for example, a diffusible gas or small molecules such as NO or ROS—is required for the dialog between gametophytes that leads to sperm discharge. In this context, the impairment in production and/or release of the molecule in one gametophyte could be rescued by diffusion of the molecule from the other nearby gametophyte. Future isolation of other mutants with defective pollen-tube reception, as well as the use of noninvasive techniques for the visualization of ROS and NO production in vivo during fertilization, should further the understanding of the peroxisome-dependent mechanisms that are critical for successful gametophyte-gametophyte communication and sperm discharge.

Supplemental Data
Supplemental Results and Discussion, Experimental Procedures, seven figures, and three tables are available at http://www.current-biology.com/cgi/content/full/18/1/63/DC1/.

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