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Two Arabidopsis AGC kinases are critical for the polarized growth of pollen tubes

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Summary

Reproduction of flowering plants requires the growth of pollen tubes to deliver immotile sperm for fertilization. Pollen tube growth resembles that of polarized metazoan cells, in that some molecular mechanisms underlying cell polarization and growth are evolutionarily conserved, including the functions of Rho GTPases and the dynamics of the actin cytoskeleton. However, a role for AGC kinases, crucial signaling mediators in polarized metazoan cells, has yet to be shown in pollen tubes. Here we demonstrate that two Arabidopsis AGC kinases are critical for polarized growth of pollen tubes. *AGC1.5* and *AGC1.7* are pollen-specific genes expressed during late developmental stages. Pollen tubes of single mutants had no detectable phenotypes during *in vitro* or *in vivo* germination, whereas those of double mutants were wider and twisted, due to frequent changes of growth trajectory *in vitro*. Pollen tubes of the double mutant also had reduced growth and were probably compromised in response to guidance cues *in vivo*. In the *agc1.5* background, downregulation of *AGC1.7* using an antisense construct phenocopied the growth defect of double mutant pollen tubes, providing additional support for a redundant function of AGC1.5/1.7 in pollen tube growth. Using the actin marker mouse Talin, we show that pollen tubes of double mutants had relatively unaffected longitudinal actin cables but had ectopic filamentous actin, indicating disturbed control of polarity. Our results demonstrate that AGC1.5 and AGC1.7 are critical components of the internal machinery of the pollen tube leading to polarized growth of pollen tubes.

Keywords: actin cytoskeleton, male gametophyte, polarity, pollen tube growth, secondary messengers, unfertilized ovules.

Abbreviations: DAB, decolorized aniline blue; DAP, days after pollination; DAPI, 4′,6-diamino-2-phenylindole; eYFP, enhanced yellow fluorescent protein; F-actin, filamentous actin.

Introduction

Double fertilization of flowering plants requires the targeted delivery of sperm by the pollen tube (Johnson and Preuss, 2002). After landing on compatible stigmatic cells, pollen germinates and the tube grows deep inside the pistil, penetrating different tissues to arrive precisely at the micropylar end of the ovule and deliver sperm. Unlike most plant cells, pollen tubes grow in a polarized rather than in a diffuse way, i.e. growth is restricted to the tip area. Tip growth is made possible through coordinated cellular activities, including a dynamic actin cytoskeleton system, targeted exocytosis, and regulated endocytosis (Hepler *et al.*, 2001).

The growth of pollen tubes necessitates both the maintenance of polarity over a relatively long distance and the ability to change the growth trajectory upon receiving extracellular cues (Hepler *et al.*, 2001). Pollen tubes growing *in vitro* usually take a straight direction, but their growth trajectory can be changed by asymmetrically placed molecules such as cAMP (Moutinho *et al.*, 2001), nitric oxide (Prado *et al.*, 2004), Ca^{2+} (Malho *et al.*, 1995), a glycosylated protein (Cheung *et al.*, 1995), or chemocyanin (Kim *et al.*, 2003). For pollen tubes growing *in vivo*, the growth trajectory is constantly adjusted to follow extracellular cues so that their sperm cargo can be successfully delivered. The

in vivo growth trajectory can be affected by both female gametophytic and sporophytic mutations (Kim *et al.*, 2004).

Cell polarization and growth is a well-studied area in metazoans, for example in epithelial cells, neuronal growth cones, and neutrophils. It requires precisely regulated Rho GTPase activities and dynamic reorganization of the actin cytoskeleton (Etienne-Manneville and Hall, 2002). Molecular mechanisms underlying polarized cell growth may have the same evolutionary origin. The plant-specific Rho GTPases (ROPs), control pollen tube polarity and growth by regulating the actin cytoskeleton and vesicle trafficking (Kost *et al.*, 1999; Li *et al.*, 1999; Fu *et al.*, 2001; Baxter-Burrell *et al.*, 2002; Cheung *et al.*, 2003). The actin cytoskeleton of pollen tubes not only facilitates organelle movement in the tube shank but may also have functions in regulated endocytosis for membrane retrieval and signaling (Staiger *et al.*, 1994; Cheung and Wu, 2004; Cardenas *et al.*, 2005).

Cell polarization and growth of metazoan cells involve precisely regulated activities of AGC kinases, which are serine/threonine kinases collectively named to include cAMP-dependent protein kinases (PKA), cGMP-dependent protein kinases (PKG), protein kinase C (PKC), protein kinase B (PKB), phosphoinositide-dependent kinase (PDK), and ribosomal protein S6 kinases (Sobko, 2006). In representative metazoan cells, extracellular stimuli are perceived at the plasma membrane, which are then transduced by the production of secondary messengers such as cAMP, cGMP, and various phospholipids. Secondary messengers activate different AGC kinases, leading to signaling relays through the phosphorylation of AGC substrates (Bogre *et al.*, 2003).

Homologs of AGC kinases have been identified in plants (Bogre *et al.*, 2003). Despite some sequence and structural differences with their metazoan counterparts, plant AGC kinases were expected to play similarly critical roles in cellular signaling. Plant AGCs were reported to regulate several important developmental processes such as auxin transport (Christensen *et al.*, 2000; Benjamins *et al.*, 2001; Friml *et al.*, 2004; Lee and Cho, 2006), biotic and abiotic responses (Rentel *et al.*, 2004; Devarenne *et al.*, 2006), blue light perception (Sakai *et al.*, 2001), and root development (Oyama *et al.*, 2002; Anthony *et al.*, 2004; Rentel *et al.*, 2004; Santner and Watson, 2006). However, the potential function of AGC kinases in the polarized growth of pollen tubes, the plant equivalent of polarized metazoan cells, has not been explored.

We report here that two closely-related Arabidopsis AGC VIIIa kinases are critical for the polarized growth of pollen tubes. *AGC1.5* and *AGC1.7* are pollen-specific genes. Pollen tubes of single T-DNA insertion mutants did not show detectable morphological changes, whereas those carrying both mutations displayed distorted morphologies, i.e. wider tubes with twists due to frequent changes of growth trajectory. Antisense downregulation of *AGC1.7* in the *agc1.5* mutant background phenocopied the growth morphology of double mutants during *in vitro* germination, further supporting the redundant function of these two AGC kinases in polar growth. Double mutants of *AGC1.5* and *AGC1.7* showed significantly compromised transmission through the male but not the female gametophyte. We used the actin marker mouse Talin (mTalin) to show that there was ectopic filamentous actin (F-actin) at each bulge in double mutant pollen tubes, although longitudinal actin cables were mostly intact. Our results demonstrate that AGC1.5 and AGC1.7 are critical components of the internal machinery of the pollen tube leading to the polarized growth of pollen tubes.

Results and discussion

The expression of the Arabidopsis AGC1.5 *subfamily is restricted to tip-growing cell types*

The Arabidopsis genome encodes 39 AGC kinases (Bogre *et al.*, 2003). Microarray data (Zimmermann *et al.*, 2004) showed that three closely related AGC VIIIa kinases had spatially restricted expression patterns: *AGC1.5* and *AGC1.7* were expressed in pollen while *AGC1.6* was expressed in root. We were interested in the potential functions of AGC kinases in pollen tube growth, so we further analyzed this AGC subfamily.

First we performed RT-PCR on RNAs extracted from diverse tissue types to confirm the expression profiles of the *AGC1.5* subfamily. According to our RT-PCR results (Figure 1a), *AGC1.5* and *AGC1.7* were pollen-specific/enriched while *AGC1.6* was detected weakly both in pollen and root. However, the nucleotide sequence of *AGC1.6* has regions of identity with that of *AGC1.7* and the primers used to amplify *AGC1.6* also amplify *AGC1.7* (data not shown), so we could not be sure if *AGC1.6* was expressed in pollen, root, or both. To further verify the expression profiles of the *AGC1.5* subfamily, we cloned the promoter sequences of the three genes in front of the enhanced yellow fluorescent protein (eYFP) reporter and generated six to eight independent single-copy insertion lines for each in the *quartet1* (*qrt1*) background. For the *pAGC1.7:eYFP* lines, the YFP signal was first detected in pollen during the bicellular stage (Figure 1b–d) and persisted in germinating pollen (Figure 1e–j) and pollen tubes (Figure 1k). All other vegetative and reproductive tissues examined did not show YFP expression (data not shown). The *pAGC1.5:eYFP* lines had the same expression profile as the *pAGC1.7:eYFP* lines (data not shown). No YFP signal was detected in the *pAGC1.6:eYFP* lines during any stage of pollen development, germination, or tube growth (data not shown). However, root hairs of the *pAGC1.6:eYFP* transgenic plants showed strong YFP expression (Figure 1l). The promoter activity of *AGC1.6* was developmentally regulated, in that the YFP signal was only detected in trichoblast cells when they were undergoing rapid root hair growth but not in trichoblast cells

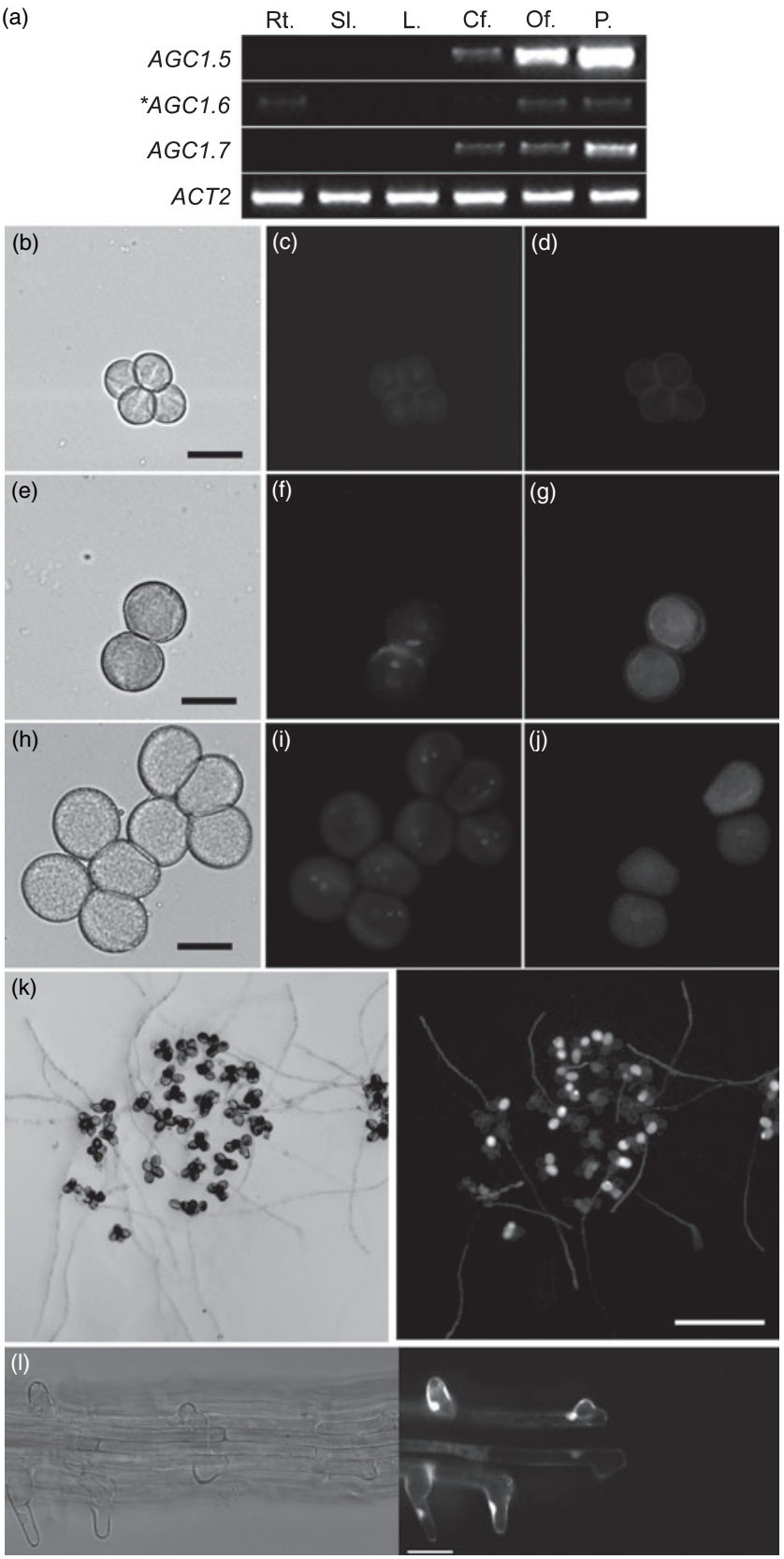


Figure 1. The expression of the *AGC1.5* subfamily is spatially restricted to tip-growing cell types.
(a) Expression profiles of the *AGC1.5* subfamily by RT-PCR. **AGC1.6* primers also amplify *AGC1.7*. Rt, roots; Sl, seedlings; L, leaf; Cf, closed flowers; Of, open flowers; P, pollen. *ACTIN2* (*ACT2*) was used as the internal control.
(b–d) *pAGC1.7:eYFP* transgenic pollen at the unicellular stage.
(e–g) *pAGC1.7:eYFP* transgenic pollen at the bicellular stage.
(h–j) *pAGC1.7:eYFP* transgenic pollen at the tricellular stage.
(k) *pAGC1.7:eYFP* transgenic pollen germinated on solid medium. Bright field and yellow fluorescent protein (YFP) channel images are shown side by side.
(l) Root hairs of a *pAGC1.6:eYFP* transgenic plant expressing YFP.
Note: *pAGC1.7:eYFP* was transformed into the *qrt1* background. Pollen from a heterozygous single-copy insertion line is shown, in which only two of the four pollen grains in a given quartet should be transgenic. The quartet shown in (e–g) broke into two dyads while preparing the slide. Parts (b), (e) and (h) are bright field images; (c), (f) and (i) were taken using 4′,6-diamino-2-phenylindole (DAPI) channels; (d), (g), and (l) were taken using YFP channels. Scale bar for (l) = 50 μm. Scale bars for (b–j) = 20 μm. Scale bar for (k) = 100 μm.

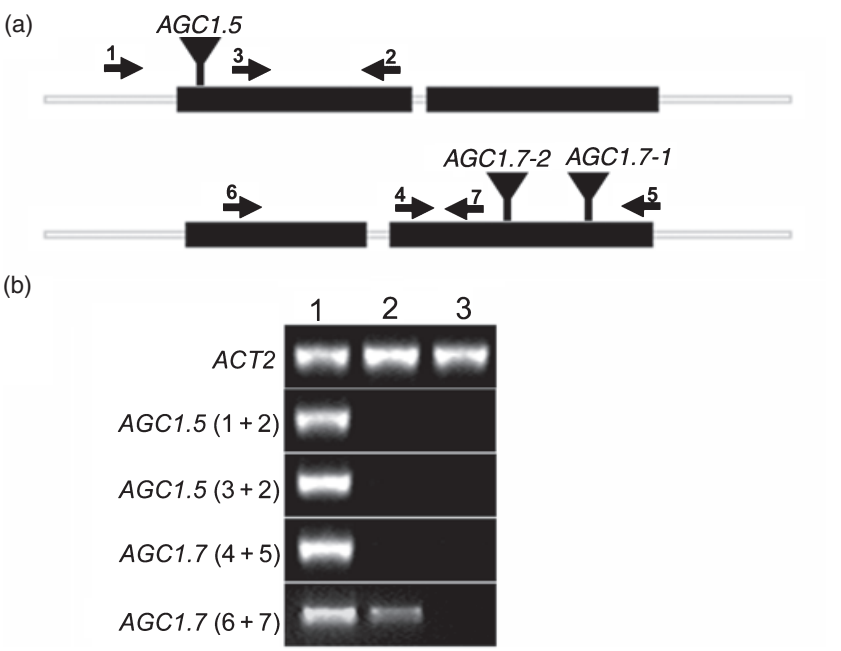
that had finished root hair growth or had just started root hair initiation (Figure 1l).

Pollen tubes and root hairs are the only types of cells in angiosperms that grow in a polar way. A large collection of transcripts accumulate in mature pollen that will be translated into proteins for germination and tube growth (Pina *et al.*, 2005). Presumably, many genes involved in this process are specifically expressed or enriched in pollen. *AGC1.5* and *AGC1.7* fit this criterion, based on the results from RT-PCR and promoter analyses (Figure 1). Their expression was spatially restricted to pollen, and they were also temporally regulated. The promoter activities of both *AGC1.5* and *AGC1.7* were detected after the bicellular stage, making them 'late' genes that are potentially involved not in pollen development but in germination and/or tube growth.

Characterization of AGC1.5 *and* AGC1.7 *T-DNA insertion mutants*

To understand the potential roles of AGC1.5 and AGC1.7 during pollen tube growth, we obtained one T-DNA insertion line for *AGC1.5* (SALK_073610, herein *agc1.5*) and two T-DNA insertion lines for *AGC1.7* (SALK_140378 and CS807313, herein *agc1.7-1* and *agc1.7-2* respectively).

The *agc1.5* line has the T-DNA insertion in the first exon, 10 nucleotides downstream of the start codon (Figure 2a).



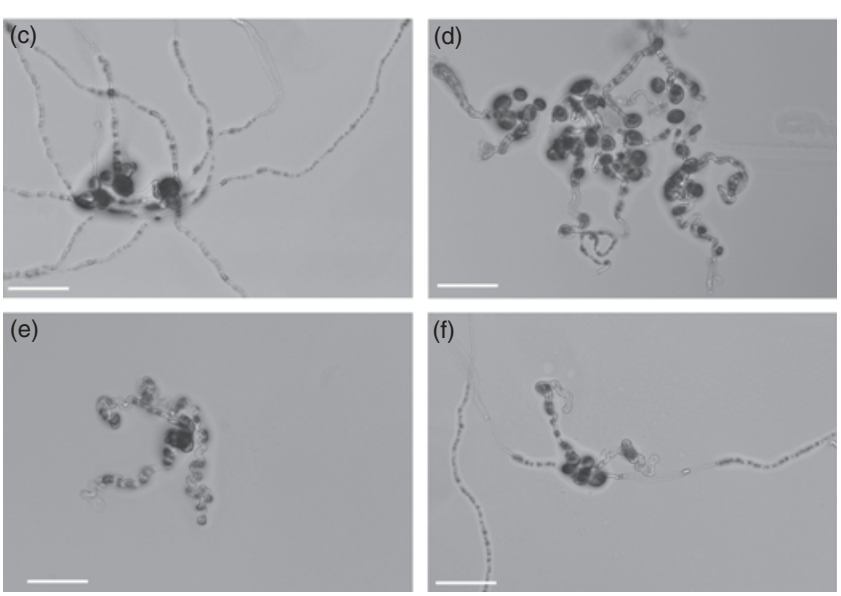


Figure 2. Characterization of T-DNA insertion lines for *AGC1.5* and *AGC1.7* and *in vitro* pollen germination.
(a) Diagram of T-DNA insertion lines in *AGC1.5* (*agc1.5*) and *AGC1.7* (*agc1.7-1* and *agc1.7-2*). Arrows indicate primers used in the transcript analysis shown in (b).
(b) Transcript analysis of *AGC1.5* and *AGC1.7* in wild type (sample 1) and in the double mutants *agc1.5*; *agc1.7-1* (sample 2) and *agc1.5*; *agc1.7-2* (sample 3). The RNAs were extracted from open flowers. *ACT2* was used as the control.
(c) *In vitro* germination of *qrt1* pollen.
(d) *In vitro* germination of *agc1.5*; *agc1.7-1* homozygous pollen.
(e) *In vitro* germination of a quartet homozygous for both *agc1.5* and *agc1.7-2*.
(f) *In vitro* germination of a quartet homozygous for *agc1.7-2* but heterozygous for *agc1.5* (*agc1.5*+/–; *agc1.7-2* –/–). In this case, two pollen tubes carry null mutations at both *AGC1.5* and *AGC1.7* while the other two contain a mutant *agc1.7-2* allele but a wild-type *AGC1.5* allele.
Scale bars for (c)–(f) = 100 μm.

The insertion completely abolished production of mRNA, indicating that *agc1.5* is a null mutant (Figure 2b). The *agc1.7-1* line has the T-DNA inserted in the second exon (Figure 2a), resulting in the production of a truncated mRNA (Figure 2b). Sequencing using the T-DNA-specific primer showed that the truncated mRNA potentially encodes a protein product in which the last 81 amino acids of AGC1.7, including the last 6 amino acids (KQHPFF) of the kinase domain, are replaced by a T-DNA-derived sequence of 18 amino acids (NNTLRTFLMYWGGFSFHG) before reaching a stop codon. The *agc1.7-2* line is a SAIL line that was generated in the *qrt1* background. The T-DNA insertion of *agc1.7-2* was also in the second exon (Figure 2a), and abolished mRNA production (Figure 2b), indicating that *agc1.7-2* is a null mutant.

Both T-DNA flanking sites were confirmed for all three T-DNA insertion lines, suggesting that deletions of other genes had not occurred. Homozygous mutants were obtained for all three insertion lines. Reciprocal crosses using heterozygous single mutants and wild type were carried out to determine if transmission was affected by the disruption of single AGCs. As shown in Table S1, both female and male transmission were normal for all three mutant alleles, suggesting that single mutations in either *AGC1.5* or *AGC1.7* had no effect on the function of pollen tubes.

Pollen tubes of the double mutants are defective in polarized growth in vitro

No developmental defects were observed in the pollen of single mutant plants using 4′,6-diamidino-2-phenylindole (DAPI) staining (data not shown) and *in vitro* pollen germination assays showed no detectable abnormalities in tube growth or morphology (Figure S1b–d), relative to wild type (Figure 2c, Figure S1a). AGC1.5 and AGC1.7 have high sequence identity and are both expressed during late developmental stages, arguing for redundant functions. Similar situations were reported for other AGC kinase pairs such as WAG1/WAG2 (Santner and Watson, 2006) and NPH1/NPHL (Sakai *et al.*, 2001). We therefore crossed *agc1.5* with either *agc1.7-1* or *agc1.7-2* to generate double mutants. We managed to obtain homozygous double mutants from both crosses by genotyping progenies of self-pollinated F_1 plants (*agc1.5*+/−; *agc1.7*+/−). However, homozygous double mutants were obtained in much lower frequencies (1 out of 108, or 1 out of 135, rather than the expected 1 out of 16), indicating that pollen grains carrying mutations at both *AGC1.5* and *AGC1.7* are functionally impaired.

No developmental defects were observed in the pollen of double mutants, just as in single mutants (data not shown). However, during *in vitro* pollen germination, pollen tubes of the double mutants showed striking morphological distortions. Wild-type pollen tubes grow along a certain axis and therefore have clearly defined apical and lateral regions with a relatively straight trajectory (Figure 2c, Figure S1b). The pollen tubes of *AGC* single mutants grew in the same way as those of the wild type (Figure S1c,d). In contrast, pollen tubes mutate in both *AGC1.5* and *AGC1.7* showed compromised polarity control, such that the distinction between apical and lateral regions was less obvious. Multiple bulges were present along the lateral region of double mutant pollen tubes during *in vitro* germination (Figure 2d–f, Movie S1), presumably due to frequent and spontaneously changed growth trajectories. All double mutant tubes were wider (14.7 ± 2.6 μm, $n = 78$) than wild-type tubes (8.7 ± 1.9 μm, $n = 76$), also indicating disturbed polarity maintenance.

Pollen of *AGC* double mutants showed no reduction in germination percentage and was not delayed in *in vitro* germination, relative to the wild type (data not shown). However, pollen tubes of double mutants not only showed reduced growth but also stopped growth much earlier than those of the wild type. Wild-type pollen tubes reached about 400 μm in approximately 6 h and kept growing to around 900 μm at 12 h. In contrast, double mutant pollen tubes only reached about 200 μm in approximately 6 h and the apical area was highly vacuolated, although the tubes were not dead as cytoplasmic streaming was still present (Movie S1).

To further demonstrate that the loss of function of both genes was required for the phenotype, we generated a binary construct expressing antisense *AGC1.7* under the control of the pollen-specific promoter *pLAT52* (Twell *et al.*,1990) (*pLAT52:asAGC1.7*). An additional *pLAT52:eGFP* expression cassette in the same binary construct enabled us to distinguish transgenic tubes from non-transformed ones. The *pLAT52:asAGC1.7*; *pLAT52:eGFP* construct was transformed into both the *agc1.5* and *qrt1* backgrounds. At least 10 independent transgenic lines in each background were obtained. Transcript analysis of transgenic lines confirmed that the transcript level of *AGC1.7* was significantly reduced by the antisense construct both in the *qrt1* and *agc1.5* backgrounds (Figure S2a). However, only transgenic pollen tubes in the *agc1.5* background (Figure S2c) and not in the *qrt1* background (Figure S2b) displayed tube widening and twisting, reminiscent of the phenotypes shown by the pollen tubes of the double mutants (Figure 2d,e). These data further support that AGC1.5 and AGC1.7 act redundantly in the polarized growth of pollen tubes.

Pollen of double mutants showed compromised competitiveness in vivo

In vitro germination results indicated that polarity was distorted in double-mutant pollen tubes. The extremely low frequency of obtaining double mutants from

self-pollinated F_1 plants suggested that pollen tubes disrupted for both genes were also defective *in vivo*. To test if this was the case, we first made reciprocal crosses between heterozygous double mutants and wild type. We focused on the heterozygous double mutant *agc1.5*+/–; *agc1.7-2*+/–, in which mutant alleles for *AGC1.5* and *AGC1.7* are null, but similar results were obtained from reciprocal crosses between *agc1.5*+/–; *agc1.7-1*+/– and wild type (data not shown). The expected segregation ratio was obtained when heterozygous double mutants were used as females (Table S1). In contrast, pollen carrying both null mutations showed a much lower transmission (Table S1), indicating that male gametophyte function was impaired.

The lower competitiveness of double-mutant pollen *in vivo* could be due to slower tube growth, a compromised response to female cues, or both. To find out which was the case, we stained pollen tubes in the pistils with decolorized aniline blue (DAB) 2 days after pollination (DAP) and analyzed seed set on homozygous double mutants and wild type. The DAB staining showed that many wild-type pollen tubes grew to the bottom of pistils (Figure 3c). Pollen tubes of double mutants germinated on the stigma in the same way as those of the wild type (Figure 3). However, few of the mutant pollen tubes reached the bottom of pistils (Figure 3d) compared with those of the wild type (Figure 3c), indicating that mutant pollen tubes had impaired growth *in vivo*. Unlike wild-type pollen tubes (Figure 3e), those of the double mutants were thick and twisted, sometimes even knotted, on the stigma (Figure 3g) and in the transmitting tract (Figure 3i). Almost all ovules in wild-type pistils were targeted by pollen tubes (Figure 3c), whereas small, undeveloped ovules (assumed to be unfertilized) were interspersed with fertilized ovules in mutant pistils (Figure 3d). The undeveloped ovules were more frequently observed in the lower part of mutant pistils (Figure 3d), but there were also significant numbers of undeveloped ovules in the upper part of mutant pistils, even though pollen tubes were available in the transmitting tract (Figure 3d,h). These results suggest that pollen tubes carrying both mutations may also have a compromised response to guidance cues.

Seed set analyses supported the conclusions drawn from the DAB staining. Although homozygous double mutants set some seeds, 31.5 $\pm$ 4.7% of the ovules (n = 4279) within mutant siliques were undeveloped (Figure 3b), in comparison to 2.7 $\pm$ 1.2% (n = 625) in the wild type (Figure 3a). The undeveloped ovules in double-mutant siliques were mainly present at the bottom of the siliques. We calculated the percentage of developed ovules in the top and bottom parts, respectively. The upper part of mutant siliques showed significantly fewer undeveloped ovules (13.7 $\pm$ 3.7%) than the lower part of mutant siliques (48.2 $\pm$ 4.2%), further indicating that mutant pollen tubes had impaired growth *in vivo*.

Interestingly, the upper half of homozygous double-mutant pistils contained a significantly higher number (13.7 $\pm$ 3.7%) of undeveloped ovules than those of wild type (1.3 $\pm$ 1.2%, n = 625). Results from aniline blue staining showed that the upper half of pistils of homozygous double mutants had small undeveloped ovules despite the available mutant pollen tubes passing through in the transmitting tract. That some mutant pollen tubes failed to exit from the transmitting tract for fertilization is suggestive of a compromised response to funicular guidance cues. However, since polarity control and response to guidance cues are inextricably associated, we cannot exclude the possibility that such a difference was also due to impaired growth of the double-mutant pollen tubes.

Pollen tubes of double mutants have a disrupted actin cytoskeleton system

The actin cytoskeleton is essential for polarized growth of pollen tubes, providing mechanical support, moving organelles, and participating in signaling events (Staiger *et al.*, 1994; Cheung and Wu, 2004; Cardenas *et al.*, 2005). To find out whether the actin cytoskeleton was disrupted in the double mutants, we generated stable transgenic lines expressing YFP-fused mouse Talin (YFP–mTalin) driven by *pLAT52* in wild type, and then crossed this marker into the double mutants. Mouse Talin has been extensively used to visualize the actin cytoskeleton in pollen tubes (Kost *et al.*, 1998; Fu *et al.*, 2001). Although high expression of mTalin can perturb the organization of the actin cytoskeleton (Ketelaar *et al.*, 2004), moderate mTalin expression was successfully used to display the dynamic actin cytoskeleton system in pollen tubes (Kost *et al.*, 1998; Fu *et al.*, 2001).

Two distinct features of the actin cytoskeleton were revealed by comparing the mTalin labeling patterns in *AGC* double-mutant pollen tubes with those in the wild type. In wild-type pollen tubes, extensive longitudinal actin cables were seen along the pollen tube shank but were excluded from the apical clear zone (Figure 4a). The expression of YFP–mTalin did not affect tube morphology in the wild type (Figure 4a). Pollen tubes of double mutants also had actin cables longitudinal to the main growth axis despite frequently changed growth trajectories (Figure 4b–d), but these actin cables also penetrated into the tip area (Figure 4b–d). A similar, although more severe, effect on actin cable structure was reported for the overexpression of constitutively active ROPs, where polarity was completely lost (Kost *et al.*, 1999; Fu *et al.*, 2001). Filamentous actin was only found in the apical region of wild-type tubes (Figure 4a), as previously reported (Fu *et al.*, 2001), and was proposed to regulate polarized growth (Fu *et al.*, 2001). In contrast, pollen tubes of double mutants had F-actin in the apical area but also underneath

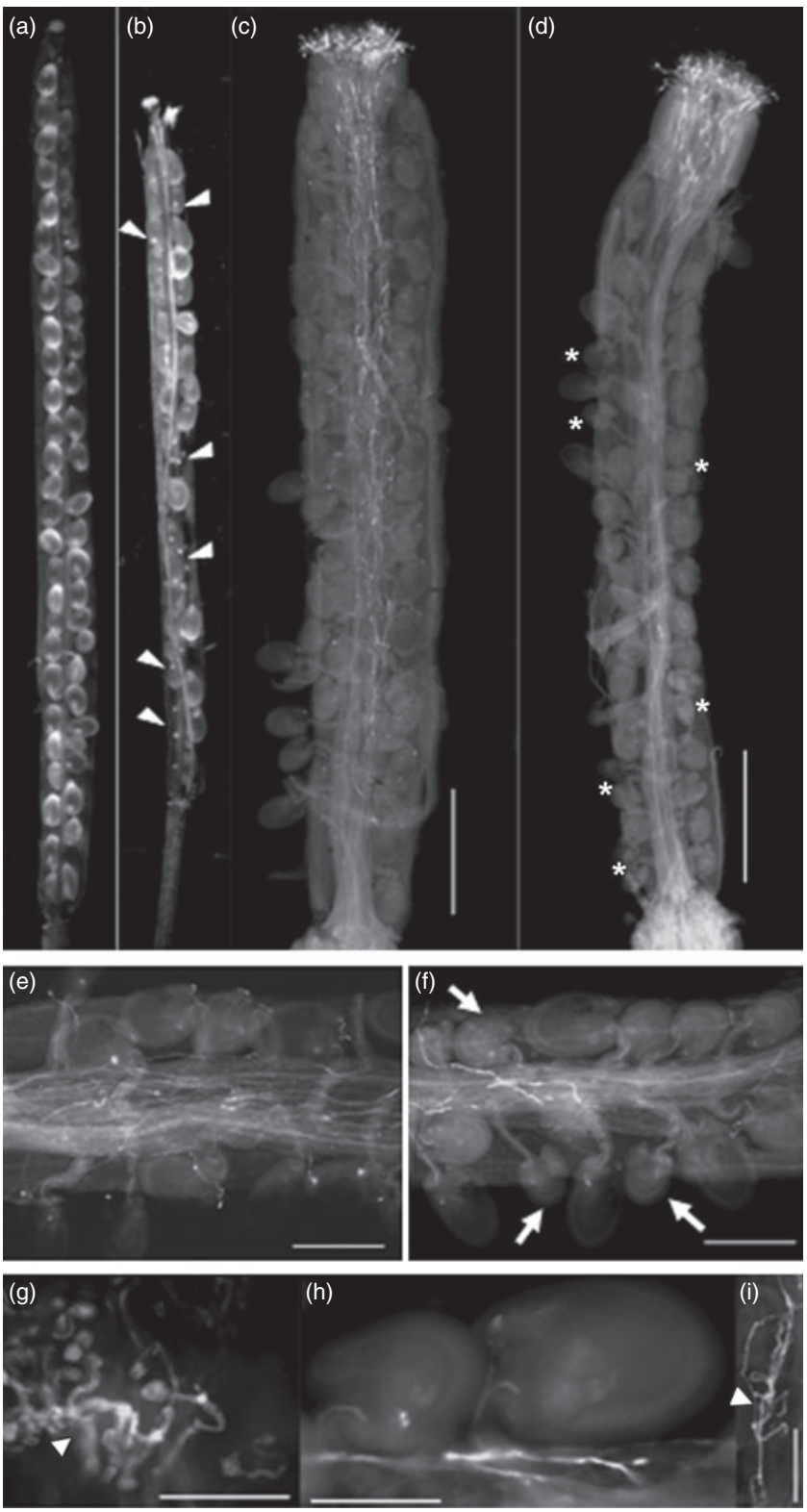


Figure 3. Pollen tubes of double mutants show impaired tube growth and probably compromised response to extracellular cues *in vivo*.
(a) Silique from a wild-type plant.
(b) Silique from an *agc1.5*; *agc1.7-1* homozygous plant. Arrowheads indicate small presumably unfertilized ovules.
(c) Wild-type pistil stained 2 days after pollination (DAP) with decolorized aniline blue (DAB); image captured using the 4′,6-diamino-2-phenylindole (DAPI) channel. Pollen tubes are seen reaching the bottom of the pistil and even ovules at the bottom part are targeted.
(d) Double-mutant pistil 2 DAP, with the same treatment as in (c). Asterisks indicate some small, presumably unfertilized, ovules. Although ample pollen germinated on the stigma, few tubes grew all the way down the transmitting tract. In addition, even in the upper part of the pistil, small ovules can be seen interspersed with fertilized ovules.
(e) Close-up of wild type pistil showing that all ovules were targeted by pollen tubes.
(f) Close-up of double-mutant pistil showing presumably unfertilized ovules despite available pollen tubes in the transmitting tract. Arrows indicate small, presumably unfertilized, ovules interspersed with fertilized ovules.
(g) Pollen tubes of double mutants on the stigma. Arrowhead indicates twisted pollen tubes.
(h) Close-up of ovules in a double-mutant pistil. Although pollen tubes are abundant in the transmitting tract, one of the two ovules shown was not targeted.
(i) Close-up of twisted pollen tubes in the transmitting tract of the double mutants. Arrowhead indicates twisted pollen tubes.
The same exposure time was used for image pairs (c)/(d) and (e)/(f) to ensure comparability. Scale bars for (c), (d) = 500 μm; scale bars for (e), (f) = 200 μm; scale bars for (g–i) = 100 μm.

lateral membrane bulges (Figure 4b–d). These lateral bulges presumably mark previous apical regions before the mutant pollen tubes changed growth trajectories. Such distorted organization of the actin cytoskeleton in pollen tubes of the *AGC* double mutants further indicated impaired polarity.

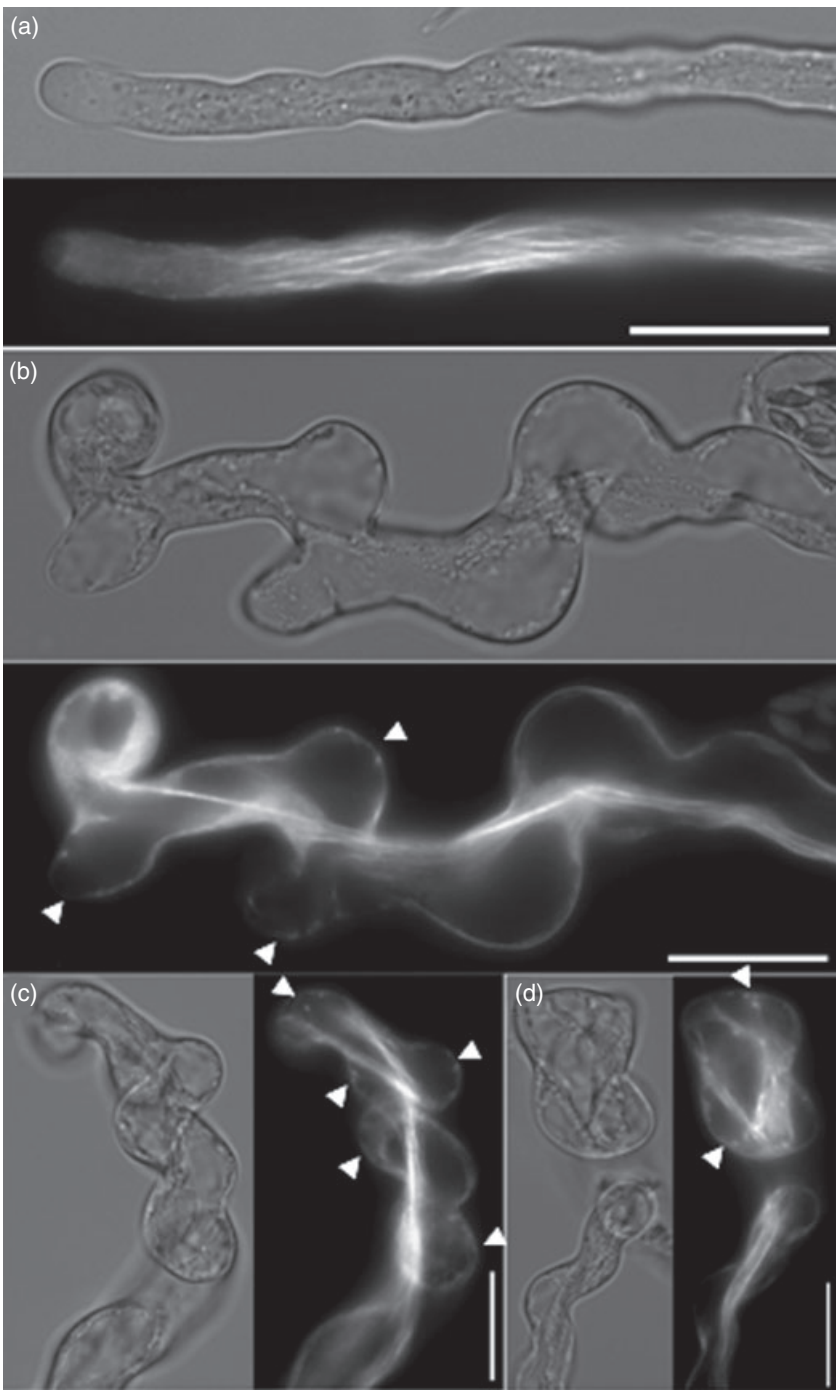


Figure 4. Filamentous actin (F-actin) was severely disturbed in double-mutant pollen tubes.
(a) A wild-type pollen tube expressing yellow fluorescent protein (YFP)–mouse Talin (mTalin), showing extensive longitudinal actin cables at the pollen tube shank and F-actin at the apex.
(b–d) Double-mutant pollen tubes expressing YFP–mTalin. Arrowheads indicate where the F-actin was detected.
Scale bars = 20 μm.

AGC1.5 and AGC1.7: signaling mediators for secondary messengers in pollen tubes?

We showed that two plant AGC kinases are critical for the polarized growth of pollen tubes. Although pollen tubes carrying mutations in both *AGC1.5* and *AGC1.7* were able to reach ovules, as shown by the availability of homozygous double mutants, they showed reduced and distorted growth, failed to compete with wild-type pollen tubes for fertilization, and were probably compromised in their ability to respond to female cues *in vivo*. Proteins involved in the perception of female cues might be expected to be at the pollen tube membrane, whereas AGC1.5 and AGC1.7 have no obvious membrane-targeting sequences. To find out where AGC1.5 and AGC1.7 localize in pollen tubes, we generated stable transgenic lines expressing AGC1.5 or AGC1.7 with either C-terminal or N-terminal eYFP fusions, driven by *pLAT52*. The control was eYFP expression alone.

The YFP signal was detected in the cytoplasm in transgenic pollen tubes expressing YFP–AGC fusions (data not shown), but a western blot (Figure S3) showed that pollen from these transgenic lines had both full-length fusion protein and free YFP present. Therefore we could not use these plants to determine the subcellular location of the AGC kinases. AGC1.7 was reported to be cytoplasmic in both yeast and tobacco BY-2 cells (Zegzouti *et al.*, 2006), but several other AGC kinases were shown to be associated with membranes despite the lack of discernable membrane-targeting signals (Briggs and Christie, 2002; Lee and Cho, 2006; Zegzouti *et al.*, 2006). The subcellular localization of AGC1.5 and AGC1.7 in pollen tubes therefore requires further investigation.

Based on the kinase domain, plant and metazoan AGC kinases are homologous. Metazoan AGC kinases participate in diverse cellular processes, especially cell polarization and directional growth, which are initiated by spatiotemporal changes of secondary messengers (Jacinto and Lorberg, 2008), but it is unknown if AGC1.5 and AGC1.7 are signaling mediators for secondary messengers in pollen tubes.

Unlike the extensive studies in polarized metazoan cells, only a few reports have addressed the function of secondary messengers in pollen tubes. Pharmacological treatments have shown that cAMP and cGMP may regulate pollen tube directional growth in that local release of cAMP- and cGMP-mediated nitric oxide was able to change the growth trajectories of pollen tubes growing *in vitro* (Moutinho *et al.*, 2001; Prado *et al.*, 2004). Such a result implies a similarity between their function in pollen tubes and polarized metazoan cells. However, cAMP and cGMP are yet to be established as important secondary messengers in pollen tubes due to the absence of their cytosolic receptors and other downstream components.

Phospholipids are ideal secondary messengers because of their ability to be rapidly generated and interconverted (Meijer and Munnik, 2003). Several phospholipids, most significantly $PI(4,5)P_2$ and phosphatidic acid (PA), affect the polarization and orientation of pollen tubes (Potocký *et al.*, 2003; Monteiro *et al.*, 2005; Dowd *et al.*, 2006; Helling *et al.*, 2006), suggesting an evolutionary similarity in cell polarization and directional growth. Among metazoan AGC kinases, protein kinase Cs (PKCs), and protein kinase B (PKB) contain phospholipid-binding motifs that enable their membrane recruitment by spatiotemporally changed phospholipids (Jacinto and Lorberg, 2008). In comparison, only one plant AGC kinase (the 3-phosphoinositide-dependent protein kinase-1; PDK1) contains phospholipid-binding motifs (Bogre *et al.*, 2003). Instead, plant AGC kinases contain a 50–70 amino acid insertion within the activation loop (Bogre *et al.*, 2003), which may be involved in subcellular targeting (Zegzouti *et al.*, 2006). Such distinct sequence features imply an early evolutionary divergence of the AGC kinase family and possibly distinct regulatory mechanisms.

Experimental procedures

Plant materials and growth conditions

The *agc1.5* (SALK_073610), *agc1.7-1* (SALK_140378), and *agc1.7-2* (CS807313) lines were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). Plants were grown in a 4:1:1 mix of Fafard 4P:perlite:vermiculite under an 18-h light/6-h dark cycle at 21°C. The *quartet1-2* (*qrt1*) mutant in the Col-0 ecotype was used as the wild type In *qrt1* all four pollen grains from a meiosis remain attached together, making phenotypic comparisons easier (Johnson-Brousseau and McCormick, 2004). The antisense *AGC1.7* construct *pLAT52:asAGC1.7*; *pLAT52:eGFP* was transformed into *agc1.5* or the *qrt1* background, using floral dipping methods (Clough and Bent, 1998).

RNA extraction and RT-PCR

Total RNA from diverse tissues of Col-0 was isolated using an RNeasy Plant miniprep kit (Qiagen; http://www.qiagen.com/) according to the manufacturer's instructions. Oligo dT-primed cDNA was synthesized using Superscript® III Reverse Transcriptase with on-column DNase-treatment, as recommended by the manufacturer (Invitrogen; http://www.invitrogen.com/). The RT-PCRs for expression profiles of the *AGC1.5* subfamily were performed using primer pairs 1 (5′-GTTTCTCTGACGCATTACAATCCCTC-3′) and 2 (5′-GCTGCAGAACTCCATGACCA-3′) for *AGC1.5*, 228 (5′-TGAGCTCCCAAAGCAACAACAG-3′) and 585 (5′-GCCGTTGAAAGATTTGACAAGT-3′) for *AGC1.6*, and 6 (5′-GCTGGGTTCACCAATCACCA-3′) and 7 (5′-CGTTGACGAAGAGTCCGAAA-3′) for *AGC1.7*. Primers to amplify *ACTIN2* (*ACT2*) were described in Zhang and McCormick (2007).

The DNA manipulations

All fluorescent protein-fusion expression constructs were generated in the Gateway system (Invitrogen) using LR reactions except where noted. Entry vectors were constructed using primers 159 (5′-CACCATGGACTTAGCTTCTAAGAAGAACACA-3′) and 160 (5′-CTAAAAGTACTCGAAATCTATATACGCCGT-3′) for *AGC1.5* and 487 (5′-CACCATGCAACAAAAGCCATTGTTC-3′) and 488 (5′-CTAAAAGTATTCAAAGTCAATGTAATCAGG-3′) for *AGC1.7*. Both *AGC1.5* and *AGC1.7* were amplified using pollen cDNA as template. Pollen-specific destination vectors were described in Zhang and McCormick (2007).

The promoter:eYFP reporter constructs were generated using the Gateway system. Promoter regions were defined as the sequences upstream of the start codon to the end of the 3′ untranslated region (UTR) sequence of the closest upstream gene. Promoter sequences were amplified from Col-0 genomic DNA using the following primer pairs: 490 (5′-CACCAGCTAATGGTCAACAATGACAGATATAC-3′) and 491 (5′-TTCCATTGGCTCACGAATAACAG-3′) for the 2880-bp *pAGC1.5*, 494 (5′-CACCACTTTTAATCCAAAACCTTTACTTCC-3′) and 495 (5′-TTAACGTTGTGATTGCTGATTAAC-3′) for the 754-bp *pAGC1.6*, and 496 (5′-CACCATCGATGTATGTTCAATTAATGGTC-3′) and 497 (5′-GTTTCAATGTAGAAAGATTAAAGCTTTAT-3′) for the 943-bp *pAGC1.7*. Entry vectors were used in LR reactions with a destination vector that contains the Gateway cassette and the eYFP reporter gene (Zhang and McCormick, 2007).

Antisense *AGC1.7* was amplified using primer pair 513 (5′-CATAGGCGCGCCACCGCCGGTACTGCAAC-3′) and 514 (5′-ATCAGGCGCGCCAACAAAAGCCATTGTTCGATCC-3′), digested with

*Asc*I, and inserted into a pollen-specific binary expression vector with an additional *pLAT52:eGFP* expression cassette (YZ and SMcC, unpublished data). The *pLAT52:YFP–mTalin* construct was subcloned into a pollen-specific pCambia from a *p35S:YFP–mTalin* construct kindly provided by Shaul Yalovsky (Tel Aviv University, Tel Aviv, Israel).

All PCR amplifications used Phusion® hot start high-fidelity DNA polymerase with the annealing temperature and extension times recommended by the manufacturer (Finnzymes; http://www.finnzymes.com/) and were sequenced using an ABI 3300 sequencer. Sequences were analyzed using Vector NTI (Invitrogen). The QIAquick® PCR purification kit, QIAprep® Spin miniprep kit and Qiagen TIP-100 kit (Qiagen) were used for PCR product recovery, DNA minipreps, and DNA midipreps, respectively.

Pollen developmental stages and pollen tube growth

Transgenic pollen of different developmental stages was obtained by dissecting anthers of different sizes. Arabidopsis *in vitro* pollen tube germination was carried out as described in Boavida and McCormick (2007). The DAPI and DAB staining procedures were as described in Johnson-Brousseau and McCormick (2004).

Pollen protein extraction and western blot

Protein extraction of pollen was as described in Muschietti *et al.* (1998). Protein concentrations were measured using a nanodrop instrument (Thermo Scientific; http://www.thermo.com/) and 40 μg total protein was loaded for each sample. Protein samples were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, http://www.gelifescience.com). The membrane was incubated with mouse monoclonal anti-GFP antibody (Clontech; http://www.clontech.com/) at a dilution of 1:2000, then with a sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech), then with ECL plus chemiluminescent substrate (Amersham Pharmacia Biotech) before exposure to X-ray film for 2 min before development.

Microscopy

Siliques were photographed using a dissecting microscope (Zeiss; http://www.zeiss.com/) equipped with a charge-coupled device (CCD) camera. Images were captured using Qcapture (QImaging; http://www.qimaging.com/). Microscopic imaging was performed using an inverted Axiophot microscope (Zeiss) with either bright-field or epifluorescence optics. Images were captured using a Spot digital camera (Diagnostic Instruments; http://www.diaginc.com/), exported using AxioVision (Zeiss), and processed using Adobe Photoshop 7.0 (Adobe; http://www.adobe.com/).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Single mutants of *AGC1.5* and *AGC1.7* showed normal pollen tube growth and morphology *in vitro*.

Figure S2. Antisense downregulation of *AGC1.7* in the *agc1.5* mutant background resulted in tube widening and twisting, recapitulating the phenotype of the *agc1.5 agc1.7* double mutants.

Figure S3. Immunoblot analysis of transgenic pollen expressing YFP–AGC1.5.

Table S1. Transmission studies on AGC single and double mutants.

Movie S1. An *agc1.5 agc1.7-2* double mutant pollen tube growing in liquid germination medium.

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