Pollen Tubes Lacking a Pair of K+ Transporters Fail to Target Ovules in Arabidopsis

Yongxian Lu, Salil Chanroj, Lalu Zulkifli, Mark A. Johnson, Nobuyuki Uozumi, Alice Cheung, and Heven Sze

INTRODUCTION

Plant reproduction is the biological basis for seed propagation, which is critical for agriculture and for species preservation. In flowering plants, male fertility and successful reproduction depend on the proper development of the male gametophyte within the anther, release and transfer of the pollen grain to an appropriate pistil, and delivery of sperm cells to the female gametophyte buried within pistil tissues. Successful double fertilization depends on the penetration of female tissues by a pollen tube, which precisely delivers the male gametes to the egg and the central cell in the ovule. Although these steps leading to fertilization have been known for decades, the molecular and cellular bases of how pollen tubes sense and respond to female signals to effect tube growth and regulate directionality of tip growth are largely unknown.

Pollen tube growth and guidance have been divided into several phases. The early stages start with penetration of the stigma and growth in the transmitting tract. Later, the pollen tube grows on the surface of a funiculus and targets the micropyle of the ovule. In the synergid cell, the tube bursts to release two sperms, one of which fuses with the egg and the other of which with the central cells (Johnson and Lord, 2006). Genetic experiments suggest that the early stages are regulated by sporophytic cells, such as those found in the transmitting tract (Crawford et al., 2007). By contrast, experiments using mutations that disrupt ovule and female gametophyte development show that the late stage of pollen tube guidance is regulated by the ovule and/or the female gametophyte (Ray et al., 1997; Shimizu and Okada, 2000).

Information emitted from female cells of both the sporophyte (pistil) and the gametophyte is important for growth and guidance of the navigating pollen tube (Hulskamp et al., 1995; Ray et al., 1997; Shimizu and Okada, 2000; Higashiyama et al., 2001; Chen et al., 2007). Interestingly, tubes of pollen germinated in vitro failed to target isolated ovules. By contrast, pollen tubes that passed through the stigma and stylar cells are competent to alter their direction to target excised ovules (Higashiyama et al., 1998). Thus, stigma and stylar cells play a role in capacitating pollen tubes to respond to late guiding signals. Candidate molecules secreted from the female cells include lipids from the wet stigma of Petunia hybrida (Wolters-Arts et al., 1998), arabinogalactan protein from tobacco (Nicotiana tabacum) transmitting tissue (Cheung et al., 1995), γ-aminobutyric acid from Arabidopsis thaliana (Palanivelu et al., 2003), and chemocyanin, a small basic protein, from lily (Lilium longiflorum) (Kim et al., 2003). The cells of the female gametophyte provide cues for the final stages of tube guidance. For
instance, Torenia embryo sac with laser-ablated synergid cells failed to attract pollen tubes, suggesting a role of synergids in pollen attraction (Higashiyama et al., 2001). Okuda et al. (2009) showed that two plant defensin-like proteins from Torenia synergid cells attract and guide pollen tube growth in vitro. In maize (Zea mays), EGG APPARATUS1 expressed only by synergids and the egg seems to function in micropylar guidance (Márton et al., 2005). Genome-wide screening of genes expressed in the embryo sac predicted many small secreted proteins that could serve as late guidance cues (Jones-Rhoades et al., 2007). Thus, a current model is that multiple stage-specific chemical cues are needed to stimulate tube growth and guidance and that different signals are likely secreted by different female cells along the tube growth pathway.

Much less is known about how the male gametophyte or pollen responds to the various chemical signals in vivo. Transmembrane receptors are likely candidates, and interaction of ligand(s) with such a receptor is thought to initiate a signaling pathway that leads to tip reorientation and tip growth. Several membrane-bound receptor-like kinases are expressed in pollen (Hony and Twell, 2004), and one of these (PRK2) has been partially characterized. Tomato (Solanum lycopersicum) PRK2 was shown to bind Lat52 protein, a pollen-specific Cys-rich protein essential for pollen hydration and tube growth (Tang et al., 2002). PRK2 also binds to Le STIG, a Cys-rich secreted protein from the pistil, which stimulates pollen tube growth in vitro (Tang et al., 2004). These studies suggest that PRK2 is involved in pollen–pistil interactions, although it is unknown whether PRK2, its homologs, or other subfamilies of receptor-like kinases perceive and then transduce guidance cues.

Studies in the last few decades have focused on in vitro pollen tip growth, so considerable information has emerged regarding the roles of ROPs (Rho of plants), actin binding proteins, ion transporters, and vesicle trafficking in tube growth (Cheung and Wu, 2008). Studies of in vitro pollen tube growth have highlighted the critical roles of Ca²⁺, K⁺, and pH dynamics that accompany tip growth (Hepler et al., 2006), indicating the importance of active and spatially localized ion transporters. Analysis of the Arabidopsis pollen transcriptome revealed many transporters that are specifically or preferentially expressed in pollen (Bock et al., 2006), although only a handful of these have been functionally studied (Sze et al., 2006). For instance, a plasma membrane (PM) H⁺ pump, Arabidopsis H⁺-ATPase 3, expressed in uncellular and bicellular male gametophytes is critical for the development of the mature pollen grain (Robertson et al., 2004). K⁺ uptake mediated by a pollen-specific Shaker K⁺ channel is essential for maintaining the rate of tube growth in vitro (Mouline et al., 2002). Both Ca²⁺ influx and its efflux from the cytosol of pollen tubes affect male fertility. Mutant pollen of the PM Ca²⁺-extrusion pump, autoinhibited Ca²⁺-ATPase 9, was defective in tube growth and in sperm discharge (Schielt et al., 2004). Furthermore, mutants of a cyclic nucleotide-gated channel localized at the PM of pollen tube tips showed reduced tube growth in vitro and in the transmitting tract (Frietsch et al., 2007).

We previously observed that 14 to 18 members of a predicted cation/H⁺ exchanger (CHX) family in Arabidopsis are preferentially or specifically expressed in mature pollen grains (Sze et al., 2004), raising questions of their multiplicity and their specific functions. Here, we show that two homologs, CHX21 and CHX23, participate in pollen tube guidance. We show that these two genes are functionally redundant. Curiously, pollen carrying mutations in both genes germinates and extends a tube into the transmitting tract just like wild-type pollen; however, the tubes of such pollen failed to make a turn and reach the ovules. Excised ovules were targeted by wild-type pollen tubes but failed to attract double mutant pollen tubes. CHX23 mediated K⁺ transport and was localized to the endoplasmic reticulum (ER) in pollen tubes. Thus, localized changes in cation and pH homeostasis are proposed to be critical in the perception and/or transduction of female cues that cause a reorientation in the axis of polarity at the pollen tip.

RESULTS

Pollen Carrying a Mutation in CHX21 or CHX23 Behaved Similarly to the Wild Type

To test whether CHX genes expressed in pollen had a role in tube growth, homozygous T-DNA insertion mutants of CHX21 and CHX23 were first identified by PCR. Total RNA was extracted from pollen and seedlings and reverse transcribed. Using three sets of primer pairs (Figure 1A), CHX23 transcripts were detected specifically in pollen, but not in the seedlings of wild-type plants (Figure 1B). However, the chx23-4 mutant contained a truncated transcript corresponding to a sequence upstream of the T-DNA insertion. No transcripts were detected using primer pairs corresponding to regions spanning or downstream of the T-DNA insertion site (Figure 1B). The T-DNA is inserted in a region encoding transmembrane 9; thus, if transcribed, a protein lacking transmembrane span 10, 11, and 12 is probably not active. In wild-type plants, CHX21 transcripts were detected in RNA preparations of both seedlings and pollen. However, there were no full-length transcripts in seedlings or from pollen of either the chx21-2 (SALK) or chx21-s1 (SAIL) mutant (Figures 1B and 1C). Partial transcripts corresponding to a region upstream of the T-DNA were also detected for chx21-2 and chx21-s1. chx21-s1 is unlikely to produce a functional protein as the T-DNA interrupted a region upstream of transmembrane region 3. However, it is possible that protein produced from the chx21-2 allele could be active, as the 12 transmembrane regions would be intact. It is unclear whether a T-DNA insertion at the hydrophilic region at the C tail would interfere with CHX21-2 activity. Therefore, we conclude that chx21-s1 and chx23-4 are likely null alleles. Although the effect of the chx21-2 allele was less certain, subsequent analysis of mutants demonstrated that it is a null mutant.

We analyzed the segregation of progeny from self-fertilized chx21-s1-/- mutants, which harbored a BASTA resistance cassette in the T-DNA insertion. The progeny yielded BASTA-resistant to BASTA-sensitive seedlings at a predicted ratio of 3:1 (Table 1, experiment i). Self-fertilization of the plant chx23-4-/- produced seedlings with the following phenotype: 34 wild-type, 43 heterozygous, and 28 homozygous for chx23-4. This is near
Double Mutant Alleles of chx21-2 and chx23-4 Are Not Transmitted through Pollen

Bioinformatic analyses indicated that CHX21 and CHX23 resembled products of a gene duplication. The two genes are located on chromosomes I and II in regions that underwent segmental duplication (Sze et al., 2004), and their coding sequences are interrupted by two introns located at identical (or conserved) sites of the coding region, although CHX23 has an extra intron (Figure 1A). The N-terminal region of both proteins is predicted to have 12 transmembrane spans, which share 70% identity (see Supplemental Figure 1 online), and the C-terminal hydrophilic regions are 68% identical.

To test whether CHX21 and CHX23 are functionally redundant, we investigated gene segregation in double mutants. First, we identified plants that were chx21-2/- chx23-4/- and then examined their descendants after self-fertilization. Instead of the expected population of CHX21+/chx23-4/- chx21-2/- chx23-4/- at a ratio of 1:2:1, the observed result was close to 1:1:0 (Table 1, experiment ii). Similarly, the progeny of self-fertilized chx21-2/- chx23-4/- showed segregation distortion. No double homozygous mutants were recovered in either experiment. The results indicated a defect in one or both gametophyte(s).

To clarify whether the defect was due to male or female transmission, reciprocal crosses with the wild-type plant were made. When chx21-2/- chx23-4/- pistils were hand-pollinated with wild-type pollen, the progeny produced the genotypes CHX21+/+ chx23-4+/- and chx21-2+/- chx23-4+/- at a ratio of 1:1. However, when pistils of a male-sterile wild-type plant (designated as Wt) were pollinated with pollen from the chx21-2+/- chx23-4+/- mutant, nearly all of the progeny produced was the single heterozygous chx21-2 mutant (chx21-2+/- CHX23+/+). Mutant plants heterozygous for both genes (chx21-2+/- chx23-4+) were rarely encountered (0.8%) (Table 1, experiment iii-a). Wt flowers cannot self-fertilize as they are male impotent (Park et al., 2002). Similarly, when Wt pistils were pollinated with pollen from the mutant chx21-2/- chx23-4/-, 98% of the progeny were single heterozygous chx23-4+/- mutants (CHX21+/+ chx23-4+/-) (Table 1, experiment iii-b). Thus, gene transmission through double mutant pollen is severely compromised. As a mixture of single mutant and double mutant pollen are produced from either chx21-2+/- chx23-4+/- or chx21-2+/- chx23-4–/- plants, the genotypes of the surviving progeny indicated that pollen defective in either CHX21 or CHX23 is functional, while the pollen defective in both is not. Therefore, CHX21 and CHX23 have redundant but essential functions in pollen.

We examined whether a wild-type copy of CHX23 could restore fertility to double mutant pollen. chx21-2+/- chx23-4–/- plants were transformed with a construct that included the native CHX23 promoter, and the CHX23 open reading frame (without the stop codon) fused at its 3’-end to a green fluorescent protein (GFP) gene. T1 plants carrying the transgene were identified. In the T2 generation, 23 chx21-2+/- chx23-4–/- double homozygotes out of 114 total plants were obtained. Twenty-eight of the remaining T2 plants were wild type for CHX21, and 63 were heterozygous for CHX21. These results indicated that wild-type CHX21 alone was able to restore function to double mutant.

The expected ratio of 1:2:1 (Table 1, experiment i). These results indicated that pollen carrying a single mutation in either chx21-2-s1 or chx23-4 was functional and fertile. Furthermore, in vitro-germinated pollen from single mutant chx21-2 or chx23-4 showed tube lengths of 372 ± 7 or 364 ± 9 μm respectively, similar to the wild-type tube length of 350 ± 10 μm at 6 h. Thus, pollen carrying a mutation in either CHX21 or CHX23 showed no defect in male fertility.
pollen (Table 1, experiment iv). We concluded that the distorted gene segregation was indeed caused by mutations in CHX21 and CHX23.

**chx21 chx23 Double Mutant Pollen Is Competent in Germination and Tube Growth but Is Infertile**

To test whether a loss in male function in chx21 chx23 double mutant pollen could be due to defective pollen development, grains were examined by microscopy. Pollen grains were collected from stage 13 chx21-2/+ chx23-4/+ flowers. This plant is expected to produce equal numbers of single and double mutant pollen. All the pollen grains were comparable to wild-type grains, as they contained three nuclei, as shown by 4',6-diamidino-2-phenylindole staining (see Supplemental Figure 2A online), and the grains turned purple after Alexander staining (see Supplemental Figure 2B online). Thus, both single and double mutant pollen were viable and apparently had undergone normal pollen development.

It is possible that the single mutant pollen (chx21-2 or chx23-4) would have a competitive advantage over double mutant pollen (chx21-2 chx23-4) in delivering the sperm to the ovule and that any growth of the double mutant pollen does not manifest due to excessive pollination. To test this possibility, one pistil of a WT pistil with a mixture of single and double mutant pollen suggested that chx21-2 chx23-4 double mutant pollen was not merely slow or less competitive than single mutant pollen but that it was infertile.

We next tested if the double mutant pollen might be impaired in germination or tube growth in vivo. After limited pollination using 38 pollen grains, the length and number of pollen tubes within the transmitting tract were examined 6 h after pollination (HAP) with aniline blue staining. The number of pollen tubes at a position 0.6

---

**Table 1. Disruption of Both CHX21 and CHX23 Genes Causes Defective Male-Specific Gene Transmission**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Parent × Male</th>
<th>Genotype or Phenotype</th>
<th>Expected</th>
<th>Observed Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>chx21-s1 × self</td>
<td>Basta&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3:1</td>
<td>341:111&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ii</td>
<td>chx23-4 × self</td>
<td>chx21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:2:1</td>
<td>34:43:28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>iii-a</td>
<td>chx21-2 × chx23-4 × self</td>
<td>chx21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:2:1</td>
<td>66:56:0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>iii-b</td>
<td>chx21-2 × chx23-4 × wild type</td>
<td>chx21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:2:1</td>
<td>56:61:0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>iv</td>
<td>WT&lt;sub&gt;1&lt;/sub&gt; × chx21-2 × chx23-4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>chx21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:2:1</td>
<td>119:1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>with gCHX23-GFP × self</td>
<td>chx21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:2:1</td>
<td>60:53&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

WT<sub>1</sub>, refers to a male-sterile mutant (At5g42650) that is wild type for all other genes.

<sup>a</sup>Not significantly different from the Mendelian segregation ratio (χ<sup>2</sup>, P > 0.05).

<sup>b</sup>Significantly different from the segregation ratio 1:2:1 (χ<sup>2</sup>, P < 0.01).

<sup>c</sup>Significantly different from the segregation ratio 1:1 (χ<sup>2</sup>, P < 0.01).

---

Despite ovules being in excess of pollen, a reduction of seed set using a mixture of single and double mutant pollen suggested that chx21-2 chx23-4 double mutant pollen was not merely slow or less competitive than single mutant pollen but that it was infertile.

---

**Figure 2. chx21 chx23 Double Mutant Pollen Appears to Be Infertile.**

(A) Diagram of limited pollination. A WT pistil was pollinated with grains from either a chx21-2<sup>b</sup> or chx21-2 × chx23-4<sup>b</sup> parent. The chx21-2 × chx23-4<sup>b</sup> parent should produce a mixture of chx21 and chx21 chx23 pollen.

(B) to (D) Pods and seeds from pistils pollinated with 35 pollen grains from chx21-2 (right) and chx21-2 × chx23-4<sup>b</sup> (left) mutants.

(B) Pod size 7 d after pollination.

(C) Pistils pollinated with pollen from chx21-2 × chx23-4<sup>b</sup> produced fewer mature seeds. Pods were cleared of chlorophyll.

(D) Number of seeds from each pod. Representative results of three independent experiments. Bar = 1 mm.

[See online article for color version of this figure.]
mm below the stigma (top) was 21 or 20 in single mutant or a mixture of single and double mutant pollen, respectively (see Supplemental Figure 3 online). Furthermore, the lengths of the pollen tubes were similar for the single and double mutant pollen based on the number of tubes (10) reaching the middle of the carpel 6 HAP. These results suggested that in vivo germination and tube elongation of chx21-2 chx23-4 double mutant pollen is comparable to that of single mutant pollen.

Double Mutant Pollen Exhibit Defective Pollen Tube Guidance to Ovule in Vivo

A major challenge in studying the function of double mutant pollen from the chx21-1 chx23-4 parent is the inability to separate the mixed population of male gametophytes with two genotypes in vivo. To help distinguish the double mutant pollen from single mutant pollen, we generated a new mutant heterozygous for chx21-s1 and homozygous for chx23-4. chx21-s1 harbors a T-DNA that contains the β-glucuronidase (GUS) reporter under the control of the pollen-specific LAT52 promoter (Sessions et al., 2002). Thus, pollen carrying chx21-s1 chx23-4 can be distinguished from CHX21 chx23-4 after staining for GUS activity (see Supplemental Figure 4A online). The mutant was generated by crossing homozygous chx21-s1 with homozygous chx23-4 plants. The F1 plant chx21-s1 chx23-4 was selfed, and chx21-s1 chx23-4 progeny were identified by BASTA resistance and PCR (see Supplemental Figure 4B online).

chx21-s1 single mutant pollen and a mixture of chx21-s1 single and chx21-s1 chx23-4 double mutant pollen were then tested for in vivo tube growth in Wt pistils. At 6 or 24 HAP, the ovary was excised to remove the carpel wall and then stained for GUS activity (see Supplemental Figure 4C online). At 6 HAP, wild-type pollen from plants expressing Lat52 promoter:GUS (WtGUS) had extended tubes more than half way in the transmitting tract (Figure 3A, i). Furthermore, the ovules located in the top half of the ovary turned blue. This indicates that the soluble GUS protein had been discharged along with sperm cells by the pollen tube at the ovule (Johnson et al., 2004). Pollen from the chx21-s1 single mutant was similar (Figure 3A, ii) to that from WtGUS pollen. By 24 HAP, tubes had reached the bottom of the transmitting tract and all the ovules in the lower half of the carpel contained some blue coloring (Figure 3B, i and ii). The turning of both wild-type and chx21-s1 single mutant pollen tubes at the funiculus and to target the ovule, indicating the discharge of pollen content into the embryo sacs (Figure 3C, i and ii). Pistils that were not pollinated showed no GUS activity in the transmitting tract or the ovules (Figure 3, iv).

Interestingly, we observed that the tubes of chx21-s1 chx23-4 double mutant pollen, which stained blue, were inside the transmitting tract and were as long or longer as those of wild-type tubes at 6 HAP (Figure 3A, iii). This result confirms the previous finding (see Supplemental Figure 3 online) that double mutant pollen was competent in germination and tube extension in vivo. The tubes reached the bottom of the transmitting tract by 24 HAP (Figure 3B, iii). Yet, strikingly, none of the ovules showed GUS activity. The tubes stayed inside the transmitting tract and failed to reorient their growth to enter the funiculus, which leads to the ovule (Figure 3C, iii). Thus, male infertility in chx21 chx23 double mutant pollen is due to a failure to shift the direction of pollen tip growth at the funiculus and to target the ovule, suggesting a failure in pollen tube guidance.

Double Mutant Pollen Also Failed to Target Isolated Ovules

We reasoned that the failure of double mutant pollen tubes to enter ovules could be a secondary consequence of defects experienced by pollen tubes in the ovarian transmitting tract. We tested this possibility by allowing double mutant pollen tubes to directly interact with ovules in a semi–in vivo assay (Higashiyama et al., 1998; Palanivelu and Preuss, 2006). In this assay, we deployed a homogeneous population of double mutant pollen obtained from a rare chx21-s1 chx23-4 double mutant plant. The mutant showed signs of sterility among the progeny of self-fertilized chx21-s1/− chx23-4/− double mutant plant. The mutant showed signs of sterility among the progeny of self-fertilized chx21-s1/− chx23-4/− (see Supplemental Figure 5 online). Vegetative growth, bolting, and the inflorescence in this mutant were indistinguishable from those of wild-type plants (see Supplemental Figure 5Aii online). Over 90% of the pods were barren, and 8% of the pods contained a single fully developed seed (see Supplemental Figure 5Aii online). These seeds germinated and the plants developed vegetative growth and flowers normally; however, like the parent, they produced only five to seven seeds per plant.

Wt, pistils were pollinated with WtGUS or chx21-s1 chx23-4 pollen, and then the ovary was severed at a site just below the
Wild-type (Wt) pistils were manually pollinated with wild-type pollen. Wild-type but not double mutant pollen tubes target isolated ovules. (A) Mutant Pollen in a Semi–in Vivo Assay. 

Figure 4. Pollen Tube Guidance Was Disrupted in chx21 chx23 Double Mutant Pollen in a Semi–in Vivo Assay.

(A) Wild-type but not double mutant pollen tubes target isolated ovules. Wild-type (Wt) pistils were manually pollinated with wild-type pollen (Wtgus) and chx21-chx23 double mutant pollen. Arrows mark the micropylar region of two untargeted ovules at the center of (ii). Arrowheads mark the micropylar region of two untargeted ovules at the center of (ii). Bar = 100 μm.

(B) Scoring targeted ovules. Number of targeted ovules to total ovules of Wtgus pollen (left) or chx21-s1 chx23-4 pollen (right). Results are from 10 experiments using ~24 ovules per experiment. 

CHX23 Is Localized to Endomembranes Resembling ER in Growing Pollen Tubes

To understand the cellular role of the predicted CHX23 ion transporter, GFP-tagged CHX23 driven by the pollen-specific promoter LAT52 (Twell et al., 1990) was expressed in tobacco pollen after bombardment. We compared the fluorescent patterns of various markers (Figures 5A to 5G; see Supplemental Movie 1 online) with that of CHX23-GFP (Figure 5H; see Supplemental Movie 2 online) and found that the CHX23-GFP signal was similar to that of an ER membrane–bound protein, SYP2.1-GFP (Ishikawa et al., 2005) (Figure 5E), and an ER lumen protein, GFP-HDEL (Figure 5F; see Supplemental Movie 1 online). To verify this possibility, tobacco pollen was cotransfected with CHX23-RFP and SYP2.1-GFP or GFP-HDEL. Merged signals from the GFP and red fluorescent protein (RFP) overlapped well in confocal images (Figure 5I, 5I”, 5J, and 5J”; see Supplemental Movie 3 online). Moreover, pollen from transgenic Arabidopsis plants expressing CHX23-GFP driven by the CHX23 native promoter also showed a similar reticulate pattern (Figure 5K; see Supplemental Movie 4 online). Thus, CHX23 appears to be localized to the ER membrane in both transiently transfected tobacco pollen and stably transformed Arabidopsis pollen. Although most of the CHX23-GFP signal corresponded to reticulate ER, the possibility that active CHX23 is localized to specific ER domains and to dynamic endomembranes that interact with the ER at the pollen tube tip remains to be examined.

CHX23: A Role in pH and K+ Homeostasis

To test the predicted cation transport activity, a cDNA encoding CHX23 was cloned. We were unable to clone a full-length CHX21 cDNA that was not mutated. CHX21 cDNAs consistently contained either a base deletion or substitution that would produce a truncated or an altered protein. CHX23 CDNA was expressed in an Escherichia coli mutant (LB2003) that grows poorly on low K+ medium due to defects in three K+ uptake systems (Stumpe and Bakker, 1997). CHX23 expression enhanced bacterial growth on yeast-tryptone-mannitol (YTM) medium containing 4 mM K+ (Figure 6A). CHX23 restored growth, similar to KAT1, which encodes an inward-rectifying K+ channel (Anderson et al., 1992; Nakamura et al., 1995; Uozumi et al., 1998). These results supported the idea that CHX23 has a role in K+ transport. Using [86Rb] as a tracer for K+, we demonstrated that CHX23 mediated K+ uptake (Figure 6C). CHX23-dependent [86Rb] uptake was reduced by increasing the concentration of monovalent cations (Figure 6D). Our results suggest that CHX23 is a monovalent cation transporter with a preference for Cs+, K+, and Rb+ relative to Na+ or Li+. Rb+ or K+ (3 mM) supplementation of medium containing 1 mM K+ supported CHX23-dependent E. coli growth; however, Cs+ or Na+ supplementation did not. Thus, Rb+ can partially substitute for K+, whereas Cs+ or Na+ cannot. Therefore, K+ is the physiological substrate of CHX23. Interestingly, CHX23-enhanced cell growth was better at pH 6.2 to 6.6 than at pH 5.6 or 7.0 (Figure 6B), indicating that the transporter is pH sensitive. Although the specific mode of K+ transport is unclear, the results point to a potential role for CHX23 in regulating homeostasis of K+, H+, or both.
DISCUSSION

Two CHX Genes Critical to Pollen Tube Guidance

We identified two related pollen-expressed genes with redundant roles in male fertility. The functional redundancy of CHX23 and CHX21 is supported not only by sequence homology, but is demonstrated also by genetic analyses (Table 1) in two ways. One, pollen from single mutants of either chx23-4 or chx21-2 behaved like the wild type. However, we showed that self-fertilization of mutants homozygous for chx23-4/− and heterozygous for chx21-2 heterozygous for chx23-4/− and heterozygous for chx21-2/− produced F1 progeny with no double homozygous mutants. Similarly, self-fertilization of mutants heterozygous for chx23-4/− and homozygous for chx21-2/− failed to produce any double homozygous F1 progeny. Second, when a wild-type pistil was pollinated by a plant homozygous for chx21-2/− and heterozygous for chx23-4/−, instead of 50% double heterozygotes in the progeny, we recovered only 0.8%. Similarly, a wild-type pistil pollinated by a plant heterozygous for chx21-2/− and homozygous for chx23-4/− yielded 2.4% double heterozygotes in the progeny, instead of 50%. These results showed that CHX23 can functionally replace CHX21 and vice versa. The reciprocal crosses indicated that the function of the female gametophyte was independent of CHX21 and CHX23 activities. Severely compromised fertility was not caused by any defect in development of the pollen grains, as shown by their normal morphology and the presence of three nuclei and the purple cytoplasm after Alexander staining.

Our findings give clues regarding which phase of pollen tube growth and guidance was impaired in chx21 chx23 double mutant pollen. Pollen tube growth and guidance have been divided into six phases: (1) stigma penetration, (2) growth in the transmitting tract, (3) transmitting tissue exit, (4) funicular guidance, (5) micropylar guidance and ovule targeting, and (6) sperm release (Johnson and Lord, 2006). Using mutants carrying a pollen-specific promoter driving the GUS reporter, we showed that chx21 chx23 double mutant pollen grains are able to germinate and extend a tube in the transmitting tract, suggesting that there is no apparent defect in the early phase of tube growth and guidance. As double mutant tubes grow to the bottom of the transmitting tract and fail to turn at the funiculus, we inferred that the ability to sense and/or respond specifically to funicular and micropylar guidance cues.

In very rare cases, double mutant pollen was fertile and was able to transmit the double mutant genotype to progeny. It is possible that the double mutant pollen tube occasionally enters the microtubule of an ovule by chance. The transmission data in Table 1 show four cases where double mutant pollen was functional. We identified a single double homozygous plant, which showed wild-type vegetative growth and developed flowers and pollen, but produced only a few seeds due to pollen tube guidance defects. Nevertheless, the ability to produce a few fully developed seeds would indicate that the last step, the sperm release and union with the egg, was not impaired in the double mutant pollen.

At this time, we cannot eliminate the possibility that chx21 chx23 double mutant pollen failed to get primed properly, as pollen tubes grow into the transmitting tract during the sporophytic phase of pollen tube growth and guidance. Evidence indicates that a priming process at the early phase of tube growth is critical for subsequent competence, as it allows the pollen tubes to perceive and respond to ovule cues (Higashiyama et al., 1998; Palanivelu and Preuss, 2006). However, what priming entails is not understood. Regardless of whether the sporophytic, gametophytic, or both phases are affected by CHX21 or CHX23, our results point to a critical role for these gene products.

Figure 5. CHX23 Localized to the ER in Growing Pollen Tubes.

(A) to (H) Fluorescent microscopy images of various markers (A) to (G) and CHX23-GFP (H) expressed transiently in tobacco pollen: (A) NtPLIM2b-GFP, actin filament marker; (B) GFP-Rab5, endosome marker; (C) GFP-Rab2, Golgi marker; (D) Mito-GFP, mitochondria marker; (E) SIP2.1-GFP, ER marker; (F) GFP-HDEL, ER marker; (G) Mito-GFP, cytosol marker; and (H) CHX23-GFP. (I) to (J') Confocal microscopy images showing colocalization of CHX23 with ER markers. Tobacco pollen cotransfected with the ER marker, GFP-HDEL (I) and CHX23-RFP (I'), or SIP2.1-GFP (J) and CHX23-RFP (J'). (I') and (J') are merged images. (K) Confocal image of CHX23-GFP in Arabidopsis pollen from plants stably transformed with ProCHX23:CHX23-GFP. Bar = 5 μm.
in sensing and/or transmitting guidance cues to enable precise targeting of pollen tubes to ovules.

Cellular Role of CHX23

CHX23 tagged with GFP at the C terminus was localized to reticulate structures resembling the ER of pollen tubes when it was expressed stably under its native promoter in transgenic Arabidopsis plants. These results are similar to those found when CHX23-GFP was transiently expressed under a strong pollen promoter, Lat52, in tobacco pollen. Moreover, confocal images showed that the fluorescence signal from CHX23-FP colocalized with two separate ER markers. We also showed that GFP-CHX23 is functional, as the T2 progeny from chx23<sup>2</sup>/2 chx21<sup>+</sup>/2 plants that carried the transgene (CHX23-GFP) included plants that were homozygous double mutants (chx21<sup>2</sup>/2 chx23<sup>2</sup>/2). These results indicated that CHX23-GFP was functionally active and able to complement chx21 chx23 double mutant pollen as seen by the change in segregation ratio (Table 1). Therefore, the most straightforward interpretation is that CHX23 is localized to endomembranes that resemble ER. However, CHX23 may be regulated by posttranslational modification, and activated versus resting transporter cannot be distinguished at this time. Therefore, the possibility that active CHX23 is localized to particular ER domains or to dynamic endomembranes that interact with the ER is considered.

Functional studies indicated that CHX23 has a role in K<sup>+</sup> transport, as shown by enhanced K<sup>+</sup> uptake into an *E. coli* mutant (LB2003) expressing CHX23. We used *E. coli* as a heterologous expression system, as CHX23 failed to rescue growth of alkaline-sensitive KTA40-2 yeast mutants. Interestingly, transporters associated with endomembranes in plant cells can be functionally expressed on the PM of *E. coli* (Uozumi, 2001; Tsunekawa et al., 2009; S. Chanroj and H. Sze, unpublished data). Furthermore, K<sup>+</sup> uptake into *E. coli* was higher at pH 6.6 than at pH 5.8, indicating that CHX23 activity is pH dependent. Two related members, CHX20 and CHX17, restored growth of KTA40-2 yeast mutants on alkaline medium containing a low concentration of K<sup>+</sup> (Maresova and Sychrova, 2006; Padmanaban et al., 2007). By contrast, a PM-localized CHX13 facilitated both yeast and plant growth under low K<sup>+</sup> and acidic conditions and mediated high-affinity K<sup>+</sup> uptake in yeast at pH 4.3 (Zhao et al., 2008). Thus, different CHXs appear to have a role in K<sup>+</sup> acquisition at various pH levels. The mode of CHX23-mediated K<sup>+</sup> transport in pollen is still unclear. It is conceivable that K<sup>+</sup> transport in exchange for H<sup>+</sup> could cause a transient and localized cation and/or pH change in the ER/endomembrane lumen and in the immediate cytosolic environment.
Indicators of intracellular K⁺ and pH changes in localized areas of the pollen tube could shed light on this working idea.

It is striking that our findings of CHX23 differ in significant ways with a prior report. Song et al. (2004) reported CHX23 expression in vegetative tissues based on promoter:GUS activity, localization of GFP-tagged protein to chloroplasts, and decrease in growth and chloroplast development of plants in which CHX23 expression was suppressed by RNA-mediated interference. However, our analysis of chx23 mutant plants did not show any growth differences from the wild type (see Supplemental Figure 6 online). Furthermore, the vegetative expression of CHX23 is inconsistent with published results using promoter:GUS, RT-PCR, and whole-genome microarrays (Sze et al., 2004; Bock et al., 2006; Hruz et al., 2008). Reasons for discrepancies include the observations that Song et al. (2004) used an undefined promoter sequence for GUS analysis and ambiguous primers for RT-PCR, thus calling into question the expression data. Furthermore, the sequence of the double-stranded RNA construct is unclear, so it is uncertain which genes were silenced in the transformants. We therefore conclude that the results of Song et al. (2004) should be viewed with caution.

Polarized Tip Growth Appears to Be Unimpaired in chx21 chx23 Pollen

Interestingly, the machinery required to sustain unidirectional polarized growth appears to be fully functional in chx21 chx23 pollen, as the tubes of these pollen grew in the transmitting tract at a rate similar to wild-type pollen. According to in vitro studies, polarized tip growth shows oscillations in growth rate at the apex of the tube. Growth is also accompanied by oscillations in cytosolic [Ca²⁺] and pH in the apical region and by oscillations in Ca²⁺ influx and H⁺ influx at the tip from the outside (Hepler et al., 2006). Although the details are still lacking, these oscillatory dynamics are thought to relate to the general physical principles of self-organizing processes, in which the ion dynamics could establish positive feedback regulation loops to generate and sustain particular spatial and temporal patterns (Feijó et al., 2001). The intracellular contents of the vegetative tube cell are also polarized, with the growing tip zone filled with secretory vesicles that fuse with the PM to drive elongation. It is devoid of large organelles, such as vacuoles, although the ER was shown to oscillate into the apex of growing lily pollen tubes (Lový-Wheeler et al., 2007).

As the ER oscillates in the tube and enters the apex, it is possible that either CHX21, CHX23, or both are modified directly or indirectly by a receptor or other interacting components, thus altering pH and/or cation levels at a localized microdomain at the tip. The resulting asymmetry facilitates in one or more ways the establishment of the new polarity axis. For instance, membrane trafficking to the tube apex, which results in deposition of new membranes and new wall materials (Cheung and Wu, 2008), might be affected. Several studies indicate that actin binding proteins are regulated by pH as well as by Ca²⁺ (Wang et al., 2008) and phosphoinositides (Cheung and Wu, 2008). One scenario is that CHX21/23-mediated local pH change alters actin polymerization, similar to the effect of a PM Na⁺/H⁺ exchanger NHE1 on Dictyostelium motility (Patel and Barber, 2005). The Dd nhe1-null mutant cannot form a polarized morphology and shows reduced F-actin polymerization. Significantly, pollen-specific receptor-like kinases exist, and one of them physically interacts with a pollen-enriched Rop-GEF in pollen tubes (Zhang and McCormick, 2007), suggesting a direct link between PM receptors and the activation of Rop to maintain polarity or reset a new orientation. Identification of funicular and microplar guidance cues as well as their pollen receptors will shed more light on how these and other regulators of polarity interact with CHX21 or CHX23 to establish a new axis of polarity.

Pollen Genes Affecting Tube Guidance

To our knowledge, only a few male gametophyte mutants have shown failure in pollen tube function. We identified a double mutant where a primary defect is failed perception, response, or both to guidance cues from the ovule. Interestingly, the defect is caused by null mutations in two paralogous CHX genes that encode predicted cation/H⁺ cotransporters (Sze et al., 2004). The CHX gene family is found in all flowering plants sequenced so far (Sze et al., 2004; Hruz et al., 2008; www.Phytozome.net). Our results suggest that multiple genes resulting from gene duplication ensure completion of essential processes, such as targeting sperm delivery to the ovule. This gene multiplicity might explain why forward genetic screens have yielded a small number of male gametophyte mutants. The finding that two related cation transporters function in the perception and/or transduction of ovule cues demonstrates that members of the CHX family are involved in signaling pathways, including networks that regulate pollen tube guidance in vivo. This unexpected finding is significant in two ways: (1) it will facilitate the identification and integration of other components critical for pollen perception and response to female cues; and (2) importantly, it will shed light on the molecular mechanism of redirected polarized tip growth not only in plants, but also in other eukaryote cells that exhibit polarity.

METHODS

Plants, Growth Conditions, and Genotyping

Wild-type Arabidopsis thaliana (ecotype Columbia-0), mutants, and transgenic plants (see Supplemental Table 1 online) were grown in Miracle-Gro potting soil (Scotts) under well-controlled environmental conditions at
Molecular Cloning of CHX23 cDNA and Genomic DNA and RNA Expression

Total RNA was isolated from mature Arabidopsis pollen, and first-strand cDNA was synthesized using reverse transcriptase (Invitrogen). Primers CHX23CF and CHX23CR (see Supplemental Table 2 online) were used to amplify CHX23 cDNA. Gel-purified PCR product was cloned into Gateway pBECHX23 after the BP reaction with the pDONR221 vector (Invitrogen). Resulting clones were verified by sequencing. Compared with the predicted full-length cDNA, the cloned coding sequence lacked 24 bases at the 3’-end. Genomic CHX23, including the promoter region and the open reading frame, was PCR amplified using primers CHX23GCF and CHX23GCR with BAC clone (F3F20_3) as template. Gel-purified PCR product was cloned into pECHX23 after the BP reaction between the PCR product and the pDONR221 vector. Resulting clones were verified by sequencing.

To express CHX23-GFP under its native promoter, genomic CHX23 was cloned into pMDC107-X23 after the LR recombination reaction between pECHX23G and pMDC107, which has a hygromycin-resistant marker. To localize CHX23 in tobacco (Nicotiana tabacum) pollen, pECHX23 was recombined into a vector that contained pollen-specific LAT52 promoter and GFP. Primers LatS2 Pf and LatS2 Pr (see Supplemental Table 2 online) were used in a PCR reaction to amplify the promoter region of the pollen-specific LAT52 gene (Twell et al., 1990). The forward and reverse primers contain SacI and SpeI sites, respectively. Gel-purified PCR product and the Gateway destination vectors p2GW7F and p2GW7R were digested with SacI and SpeI, gel purified, and ligated, resulting in new vectors, pLatGW7F and pLatGW7R, respectively. Insertion of the LAT52 promoter into the new vectors was confirmed by sequencing. After the LR recombination reaction between pECHX23 and the newly constructed LAT52-driven vectors, CHX23 cDNA was cloned into new vectors that were named pLatGW7F-X23 and pLatGW7R-X23, respectively.

RNA expression was determined by RT-PCR of total RNA. Total RNA was isolated from seedlings or pollen of Arabidopsis plants using Trizol reagent (Invitrogen). Briefly, 2-week-old seedlings grown on half-strength MS plates and pollen from 6-week-old plants (Honys and Twell, 2004) were used. Isolated RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). Gene-specific primers (see Supplemental Table 2) were used for a 30-cycle PCR. Actin 11 (At5g12110) was amplified using Actin-S and Actin-AS primers to verify equivalent loading and to detect for possible contamination of genomic DNA. Amplified products were visualized by ethidium bromide fluorescence after electrophoresis on an agarose gel.

Plant or Pollen Transformation

Tobacco pollen was transfected by bombardment (Chen et al., 2002), pLatGWF-X23 and pLatGWR-X23 plasmids were prepared using the Qiagen Plasmid Midi Kit. For each bombardment, 1.25 μg DNA and 7 mg tobacco pollen were used. Pollen was transfected with NPTII6mb-GFP (Cheung et al., 2008), GFP-Rab2 (Cheung et al., 2002), GFP-Rab5, mito-GFP, GFP-HDEL (Cheung and Wu, 2007), SIP2.1-GFP (Ishikawa et al., 2005; A. Cheung and H. Wu, unpublished data), and LAT52:GFP (Cheung, 2001) to visualize actin filament, Golgi, endosome, mitochondria, ER, and cytosol, respectively. After bombardment, pollen grains were cultured on medium containing 1 mM KNO3, 1.6 mM H2BO3, 0.8 mM MgSO4, 3 mM Ca(NO3)2, 4H2O, 8% sucrose, 0.7% agarose, and 10 mM MES, pH 6.0. After a 4-h incubation, pollen tubes were observed by microscopy.

Arabidopsis plants were transformed via Agrobacterium tumefaciens using the floral dip method (Clough and Bent, 1998). Agrobacterium strain GV3101 was transformed with the binary vector pMDC107-X23 via electroporation, and transformants were selected on Luria-Bertani plates with gentamicin (50 μg/mL) and kanamycin (50 μg/mL). For the molecular complementation, chx21-2/s1/+ chx23-4/+ plants were transformed with the construct gCHX23-GFP in the pMDC107-X23 vector. Sixteen T1 transformants were selected by resistance on half-strength MS plates containing hygromycin (30 μg/mL). Two-week-old hygromycin-resistant plants were genotyped by PCR methods using gene-specific primers. Three chx21-2/+ chx23-4/+ transformants were self-fertilized, and the T2 population was selected on hygromycin-containing plates and then tested by PCR for the segregation of the CHX21 gene only. The resulting chx21-2/+ plants were further tested with gene-specific primers of the CHX23, chx23-4, and CHX23-GFP transgenes (see Supplemental Table 2). Finally, it was confirmed that all of the chx21-2/+ chx23-4/+ plants carried the CHX23-GFP transgene. As the transgene in T1 is heterozygous, the expected genotype of the hygromycin-resistant T2 population of CHX21, chx23-4, and chx21-1 would be 3:5:2.

Pollen Assays

To test pollen fertility, pollen from stage 13 flowers was collected and counted under a stereoscopic zoom microscope (Nikon SMZ1000). Then, grains were dabbed onto the stigma surface of Wti, a male-sterile line (Park et al., 2002) that has wild-type CHX21 and CHX23. Seven days after pollination, pods were examined by stereoscopic zoom microscopy. Seeds within pods were visible and scored after depigmentation by 70% ethanol. Pods were opened and imaged.

To view pollen tubes growing inside transmitting tract, pistils were given limited pollen grains. Six hours later, the pistil was cut and fixed in 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) and examined by differential interference contrast microscopy.

Relative GUS activity in flowers, pistils, or pollen tubes was determined after incubating tissues in GUS reaction mixture at 37°C for various periods (30 min to overnight). The reaction consists of 50 mM sodium phosphate, pH 7.2, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.2% Triton X-100, and 2 mM X-Gluc. Images were taken using a Nikon microscope (SMZ1000) or a Nikon microscope with differential interference contrast (Eclipse E600).
Microscopy
Fluorescence images of tobacco pollen were taken using a Nikon E800 microscope linked to a CCD camera (Spot; Diagnostic Instruments). GFP signal was imaged using an excitation filter (460 to 500 nm; D505) and emission filter for 510 to 560 nm. Confocal images of pollen were taken with a Zeiss LSM 510 laser scanning confocal microscope. Images of GFP and RFP were observed using multitrack excitation wavelengths of 488 and 543 nm, and emission at BP 505 to 530 nm and LP 560 nm, respectively. For stably transformed Arabidopsis plants, 10 independent T3 lines were examined. Single-channel GFP confocal images were taken using excitation and emission wavelengths of 488 nm and LP 550 nm, respectively.

Functional Studies in Escherichia coli
Arabidopsis CHX23 was PCR amplified with primers AtCHX23-BgII-SE and AtCHX23-XhoI-AN (see Supplemental Table 2 online) using pECHX23 as template. The PCR product was isolated from agarose gel using a PCR DNA purification kit (Iliusa, GE Healthcare) and then inserted into the multiple cloning site of pPAB404 (Buurman et al., 1995). The ligation mixture was used to transform E. coli XL10 Gold, and transformants were plated on Luria-Bertani medium with ampicillin and 0.5% yeast extract, and 100 mM mannitol at pH 7.0. Cells were washed and incubated at 37°C overnight. To confirm the sequence, colony PCR and sequencing were performed. The resulting plasmid pPAB404-CHX23 was used to transform E. coli strain LB2003 (F-, thi, lacZ, gal, rha, ΔkdpFABC5, trkD1, ΔtrkA), which lacks three K+ uptake systems, Trk, Kup, and Kdp (Stumpe and Bakker, 1997). Growth of transformed strains was monitored by changes in cell density at OD600 using a microplate reader. Transformants were cultured in YT medium supplemented with 30 mM KCl and 50 μM/μl ampicillin (Uozumi, 2001; Zulkifli et al., 2010). Basal YT medium consisted of 1% tryptone, 0.5% yeast extract, and 100 mM mannitol at pH 7.0. Cells were washed three times with basal YT medium to reduce [K+] to ~4 mM and normalized to an A600 of 0.5. Cells (20 μL/well) were added to wells containing 180 μL of YT medium. Different concentrations of alkali cations were included, and mixtures were osmotically balanced with mannitol. The final assay mixture contained cells at an OD600 of 0.4, 1 mM RbCl (0.5 μCi/mL 86Rb), 0.5 mM IPTG in SGM, 10 mM MES, and 10 mM MOPS adjusted to pH 6.2 with Arg. Different concentrations of alkali cations were included, and mixtures were osmotically balanced with mannitol. To start, cell suspension was added 30 s before carrier-free 86Rb was introduced. Transport was stopped by adding 3.5 mL wash buffer to each tube followed by filtration and two additional rinses.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers At2g31910 (CHX21) and At1g05580 (CHX23).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Comparison of CHX21 and CHX23.
Supplemental Figure 2. Images of Stained Pollen Grains from Wild-Type and Mutant (chx21-1/2 chx23-2/3) Plants.
Supplemental Figure 3. In Vivo Germination and Tube Growth of chx21 Single and chx23 Double Mutant Pollen Are Similar.
Supplemental Figure 4. Generation of chx21 chx23 Double Mutant Pollen Carrying a GUS Reporter.
Supplemental Figure 5. A Rare chx21-s1/2 chx23-4/5 Double Homozygous Plant.
Supplemental Figure 6. Vegetative Growth of the chx23 Mutant Was Similar to That of the Wild-Type Plant.
Supplemental Table 1. Arabidopsis Plant Lines and Bacterium Used in This Study.
Supplemental Table 2. Primers Used in This Study.
Supplemental Movie 1. Z-Stack Confocal Images of Tobacco Pollen Tubes Expressing GFP-HDEL.
Supplemental Movie 2. Z-Stack Images of Tobacco Pollen Tubes Expressing CHX23-GFP.
Supplemental Movie 3. Z-Stack Images of Tobacco Pollen Tubes Expressing CHX23-RFP and SIP2.1-GFP.
Supplemental Movie Legends.

ACKNOWLEDGMENTS
We thank Ravi Palanivelu (University of Arizona, Tucson) for generously hosting Y.L., for suggestions on the semi-in vivo assay, and comments on this work. We thank Guoying Wang (University of Maryland, Baltimore) for help cloning Lat52 vectors and J.F. Harper (University of Nevada), and Zhongchi Liu (University of Maryland, College Park) for valuable comments. We thank Kei Nanatani (Tohoku University) for experiments with E. coli. This work is supported in part by the National Science Foundation (IBN0209788) and U.S. Department of Energy Office of Basic Energy Sciences, Division of Chemical Sciences, Geosciences and Biosciences Division DEFG0207ER15883 to H.S.;
REFERENCES


Transporters Affect Pollen Tube Guidance


