ABSTRACT

Title of Document: POLLEN TUBES FAIL TO TARGET OVULE IN THE ABSENCE OF TWO CATION/PROTON EXCHANGERS IN ARABIDOPSIS

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Flowering plant reproduction requires precise delivery of the sperm cells to the ovule by a pollen tube. Guiding signals from female cells are being identified, though how pollen senses and responds to those cues are largely unknown. Here I provide genetic evidence that two predicted cation/proton exchangers expressed in Arabidopsis pollen play essential roles in pollen targeting of ovules.

Male fertility was unchanged in single *chx21* or *chx23* mutant pollen; however, male-specific gene transmission was blocked in *chx21chx23* double mutant. Wild-type pistil provided with a limited amount of pollen containing a mixture of single and double mutant produced ~60% less seeds compared to that produced with *chx23* single mutant pollen, indicating that *chx21chx23* pollen is infertile. The double mutant pollen, visualized by a pollen-specific promoter-driven GUS activity, germinated and extended a tube down the transmitting tract, but the tube failed to turn and target an ovule. Unlike

wild-type pollen that targeted isolated ovules in a semi-*in vivo* assay, tube guidance in *chx21chx23* pollen was compromised.

As a first step to understand the cellular and molecular bases of tube guidance, membrane localization and activity of CHX23 was determined. GFP-tagged CHX23 was localized to endomembranes, predominantly endoplasmic reticulum (ER), in elongating pollen tubes. Furthermore, expression of CHX23 in *E. coli* resulted in enhanced K^+ accumulation at alkaline pH, suggesting a role for CHX23 in K^+ acquisition and pH homeostasis.

Based on these studies and observations by others that ER oscillates and enters the apex, a simple model is proposed: Modification of localized pH by CHX21 or CHX23 enables pollen tube to sense female signals and respond by shifting directional growth at the funiculus and micropyle to target pollen tip growth towards the ovule.

POLLEN TUBES FAIL TO TARGET OVULE IN THE ABSENCE OF TWO CATION/PROTON EXCHANGERS IN ARABIDOPSIS

By

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To my parents, my wife Guoying and my daughter Maggie

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ABBREVIATIONS

ABP	Actin-binding protein
AC	Antipodal cell
ACA	Auto-inhibited Ca^{2+}-ATPase
ADF	Actin-depolymerizing factor
BCECF	2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
CA	Constitutive active
cAMP	Cyclic adenosine monophosphate
CBL	Calcineurin B-like proteins
cDNA	Complementary deoxyribonucleic acid
CDPK	Ca^{2+}-dependent protein kinase
CHX	Cation/proton exchanger
CN	Central cell nuclei
CNGC	Cyclic guanine nucleotide-gated channel
CPK	Calmodulin-domain protein kinase
DAPI	4'-6-Diamidino-2-phenylindole
DIC	Differential interference contrast
DN	Dominant negative
DNA	Deoxyribonucleic acid
EC	Egg cell
ECA	Endomembrane-type Ca^{2+}-ATPase

ER	Endoplasmic reticulum
FM4-64	*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide
GAP	GTPase-Activating Protein
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide-exchange facto
GFP	Green fluorescence protein
GUS	β-Glucuronidase
HAP	Hours after pollination
KHA1	K^+/H^+ antiporter 1
mRNA	Messenger ribonucleic acid
Nc	Nuclei
NHE	Na^+/H^+ exchanger
NHX1	Na^+/H^+ exchanger 1
ORF	Open reading frame
PCD	Programmed cell death
PCR	Polymerase chain reaction
PM	Plasma membrane
PVC	Prevacuolar compartment
RFP	Red fluorescence protein
RIC	ROP-interactive CRIB-containing proteins
RLK	Receptor like kinase

RNA	Ribonucleic acid
ROP	Rho-related protein from plants
RT	Reverse transcription
SIP	Small intrinsic protein
ST	Rat sialyltransferase
SY	Synergid cell
T-DNA	Transfer DNA
TGN	Trans-Golgi network
Wt	Wild type
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acidacidcidcyclohexylammonium salt

I. PROJECT BACKGROUND

A. LITERATURE REVIEW

1. Sexual reproduction in flowering plants

Reproduction is the biological basis for crop seed production and for species preservation (Buchanan et al., 2002). The life-cycle of flowering plants alternates between sporophytic and gametophytic generations. Reproduction in most flowering plants is sexual, involving meiosis and mitosis that produce the two opposite gametes and then fertilization, fusion of the two gametes. However the male and female gametes develop in physically separate organs, so the precise delivery of the male gamete to the egg and central cell requires multiple regulated steps which are poorly understood at the cellular and molecular level.

1-1. Male gametophyte development. The male gametophyte, pollen, is developed inside the anther tissue. After two consecutive meiosis, each diploid pollen mother cell produce four haploid microspores, then each microspore goes through two mitosis to produce a tricelluar pollen grain (or pollen tube at the later stage) that includes two small sperm cells inside a big vegetative cell (McCormick, 2004; Borg et al., 2009). The first mitosis is asymmetric, produce two daughter cells with distinct destiny: the determinate vegetative cell that stops division and the generative cell that undergoes a second division to produce the two sperm cells (Fig. I-1). A somatic and non-germ line expressed repressor was proposed to repress germ cell-specific genes in the non-germ line cells (Haerizadeh et al., 2006). After the asymmetric division, an F-box

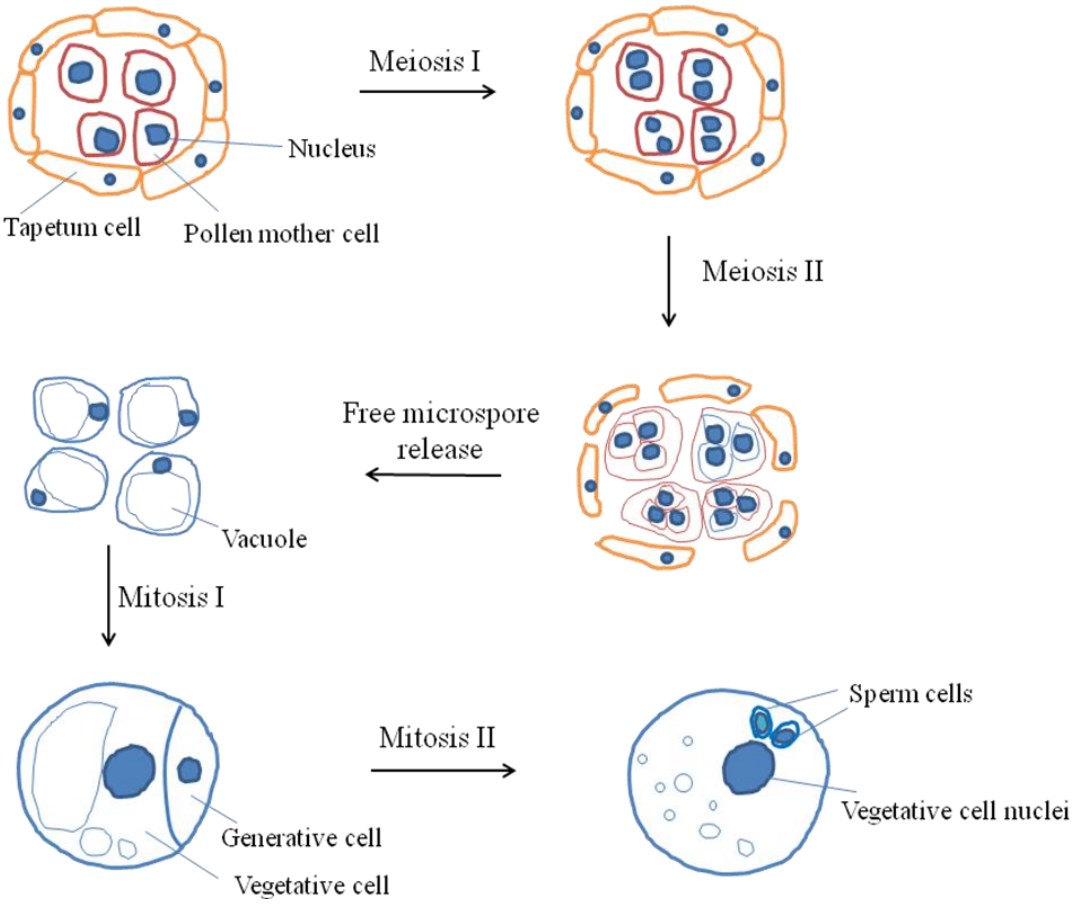


Fig. I-1. Male gametogenesis in flowering plants

The process described here starts with pollen mother cell and ends with mature pollen grain. (Modified from McCormick, 2004)

protein expressed in the generative cell, but not in the vegetative cell, was shown to promote cell division by degrading the cyclin-dependent protein kinase inhibitor (Kim et al., 2008). But overall the cellular and molecular mechanisms controlling pollen development is poorly understood (Borg et al., 2009).

1-2. Female gametophyte development. The female gametophyte (FG), the embryo sac, develops and is deeply buried inside the female organ, pistil. In Arabidopsis, one diploid megaspore mother cell produces four haploid megaspores, three of which undergoes programmed cell death. The surviving spore undergoes three consecutive mitosis and produce eight cells. After cell differentiation, a mature embryo sac forms that contains 4 different types and totally seven cells: one egg cell, two synergid cells that flank the egg cell, one polar central cells and three antipodal cells (Fig. I-2) (Yadegari and Drews, 2004). The cellular and molecular mechanisms that regulate the cell division and specification are poorly understood. Auxin gradient resulted from location-specific auxin synthesis, rather than auxin efflux was shown to determine cell fate inside the developing FG (Pagnussat et al., 2009). Thus, auxin is a plausible major morphogen for plant development not only in the sporophytic tissues, but also in the gametophtyic generation (Benkova et al., 2009).

1-3. Fertilization. One of the defining features of flowering plants is double fertilization, in which twin sperm fertilize two female gametes, the egg cell and central cell, respectively. One central question in plant reproduction is: how do the spatially separated sex cells meet and fuse? Double fertilization can be divided artificially into several successive steps: 1) pollen germination on the compatible stigma; 2) Guided

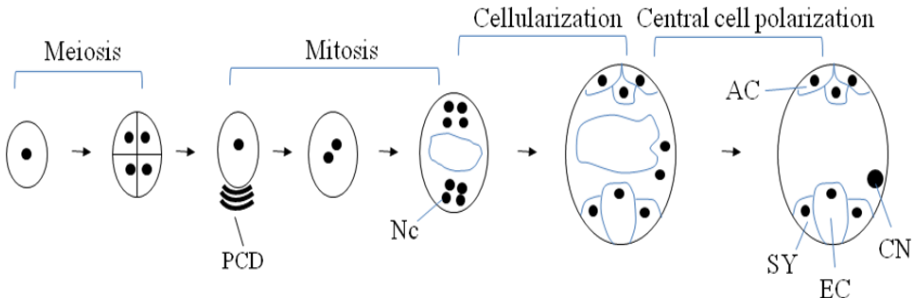


Fig. I-2. Female gametogenesis in Arabidopsis.

The process described here starts with megaspore mother cell and ends with mature embryo sac inside ovule. AC, antipodal cell; CN, central cell nucleus; EC, egg cell; Nc, nucleus; PCD, programmed cell death; SY, synergid cell. (Modified from Yadegari and Drews, 2004).

pollen tube growth that accurately deliver the sperm cells into an embryo sac; 3) sperm discharge; 4) sperm migration to the egg and central cells, respectively, and 5) gamete recognition and fusion. In the first chapter, I will review the current knowledge about pollen germination and then focus on step 2), of how the pollen might achieve the directed growth that finally target an ovule after traveling a relatively long distance (Fig. I-3). Major results of functional studies will be summarized, and questions that remain will be highlighted.

2. Pollen germination

Pollen germination is the process by which the pollen hydrates and establishes the single polar tube growth site, after which the protruding tube breaches the cell wall and emerges from the pollen grain.

2-1. Pollen hydration.

After pollination, pollen landing on the stigma interacts with stigma cells. For species that prevent inbreeding, self-incompatibility response (SI) will occur to stop pollen tube penetrating into the stigma or stop tube growth at a late stage (Takayama and Isogai, 2005). Once landed on stigma, pollen grain will hydrate, changing from the desiccated and metabolically quiescent state to active state (Edlund et al., 2004). But the mechanism (s) regulating pollen hydration is not clear. Water channels may be involved in the regulated release of water from the stigma to pollen, as evidenced by the expression of aquaporin in Brassica stigma (Dixit et al., 2001; Bots et al., 2005). Further studies are needed to identify the biological function of these aquaporins. Nevertheless, exquisite temporal and spatial regulation of the pollen hydration is critical, as evidenced

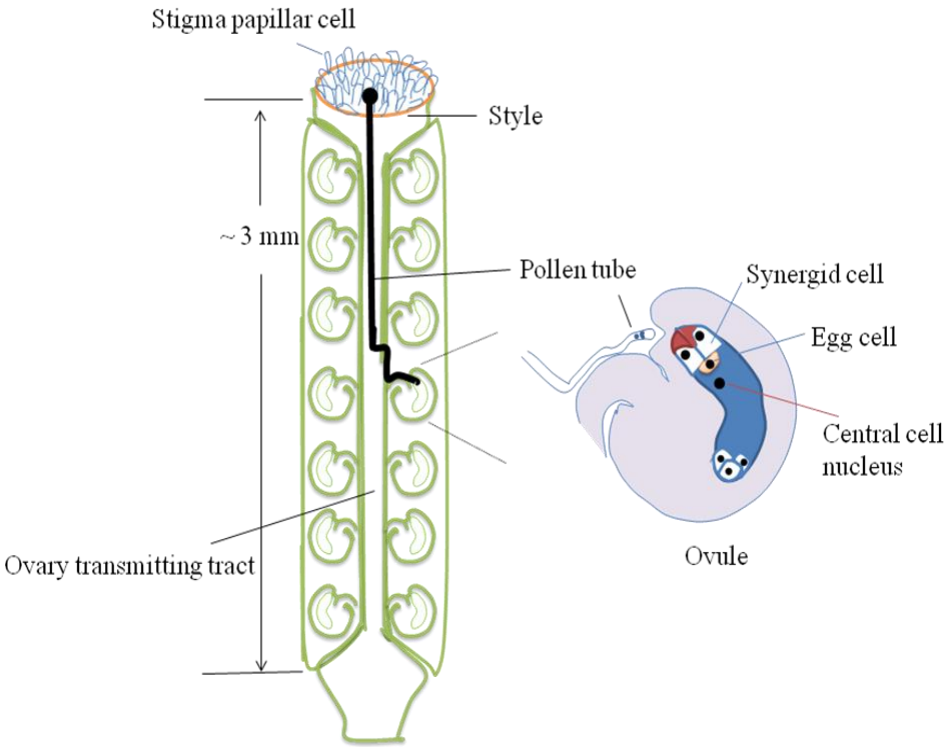


Fig. I-3. Pollen tube grows toward the ovule

Pollen tube is exaggerated to be easily seen. Pistil length is from experimental observations. (Modified from Johnson and Lord, 2006).

by the studies on *raring-to-go* (Johnson and McCormick, 2001) and *fiddlehead* (Lolle and Cheung, 1993) mutants. In the Arabidopsis *raring-to-go* mutant, pollen germinate precociously in anther and so the plants are male-sterile (Johnson and McCormick, 2001). When applied to the *fiddlehead* mutant plant, wild type pollen hydrate and germinate on the vegetative tissue and non-reproductive floral organs (Lolle and Cheung, 1993). In both mutants, communication between pollen and stigma cells was circumvented and regulation of pollen hydration was broken, resulting in disastrous consequence.

Pollen coat lipids and proteins play important role in pollen hydration. In Arabidopsis pollen, disruption of long-chain lipid synthesis resulted in prohibited pollen hydration and male sterility (Preuss et al., 1993), while addition of purified triacylglycerides restored hydraulic conduct between pollen and stigma and normal pollen hydration (Wolters-Arts et al., 1998). In the *fiddlehead* mutant, the gene that encodes a β-ketoacyl-CoA synthase required for long-chain lipid synthesis is defective, resulting in enhanced pollen hydration (Lolle and Cheung, 1993). These results suggest that pollen coat long-chain lipid plays critical role in signaling the stigma cells to release water, but the cellular and molecular mechanism is not clear. Beside lipid molecules, pollen coat protein also involves in regulating pollen hydration. In the Arabidopsis *grp17* mutant, production of the wild type protein that harbors the oleosin domain was disrupted. Mutant pollen showed severely delayed commencement of hydration and impaired ability to compete with the wild type pollen (Mayfield and Preuss, 2000). As Brassicaceae species have quite similar pollen coat lipids, but quite divergent GRP proteins, it is proposed that lipids may play a role in regulating water transfer from female cells to

desiccated pollen, while proteins involve in species-specific pollen-stigma recognition (Edlund et al., 2004).

Hydration induces global changes inside pollen that transforms a pollen grain from non-polar to a polar cell. Some major changes include polarization of actin filament toward the germination site (Heslop-Harrison and Heslop-Harrison, 1992), actin filament wraps around the nuclei (Heslop-Harrison and Heslop-Harrison, 1989), the reorientation of the vegetative nucleus so that it enter the pollen tube before the sperms (Lalanne and Twell, 2002), and the selection of the site on the plasma membrane (PM) for the polar delivery of new membrane and cell wall materials. But the questions of what is the initial signal induce the cell polarity and how the signal is finally perceived and transduced to select the single site for tube protrusion is not clear.

2-2. Polar site selection.

Several molecules have been proposed to initiate polarity, include water (Lush et al., 1998) and lipids (Wolters-Arts et al., 1998). When tobacco pollen was put into stigma exudates or purified lipids, pollen tube always protrude from the pollen side next to an aqueous interface, suggesting water provides a directional cue to select the polar site. It is possible that water and lipids work together to send the directional signal to germinating pollen, by creating a water gradient that is strong enough to be sensed by the pollen at one side, but not too much to engulf the whole pollen grain.

It is thought that an initial polar signal induces the polar localization of Rac/Rop GTPase activity to the germination site, which in turn serves as a “master control” and

regulates other cellular components like ions gradients, actin filament dynamics and vesicle trafficking (Nibau et al., 2006; Lee and Yang, 2008). Pollen tube then starts the single-minded long journey within the relatively short pistil tissue to deliver the sperms to the ovule.

3. Pollen tube growth

Pollen tube grows by tip growth (Steer and Steer, 1989; Hepler et al., 2001), which allows the pollen vegetative cell to travel relatively enormous distance to provide the sperm for double fertilization. Further, this tip-localized growth allows the growing tube to communicate and response to the female cell environment effectively. Growing pollen tube restricts cytoplasm to the apical region of the tube by forming large vacuoles and callose plugs at the basal end, maintaining a relatively consistent cytoplasm volume. In some species, pollen tubes show astounding growth rates, like lily pollen tubes growing in vitro elongate at a speed of 250 nm/s (Pierson et al., 1996; Messerli et al., 2000). In vitro growing pollen tubes show oscillatory patterns, with rapid and slow growth phases (Feijo et al., 2001). The cytoplasm exhibits vigorous streaming motion and shows a “reverse fountain” pattern. Pollen tube cytoplasm is polarized, including an apical “clear” zone containing small vesicles but excludes large organelles, the subapical zone that is rich in organelles like Golgi, the nuclear zone that contains the vegetative nucleus and the two sperms, and the vacuolization and callosic plug zone (Fig. I-4) (Steer and Steer, 1989). Small vesicles in the apex are thought to deliver new membrane and wall materials to the growing site to sustain the fast tip growth. Recent studies show that major cellular components are involved in pollen tip growth, including ion dynamics,

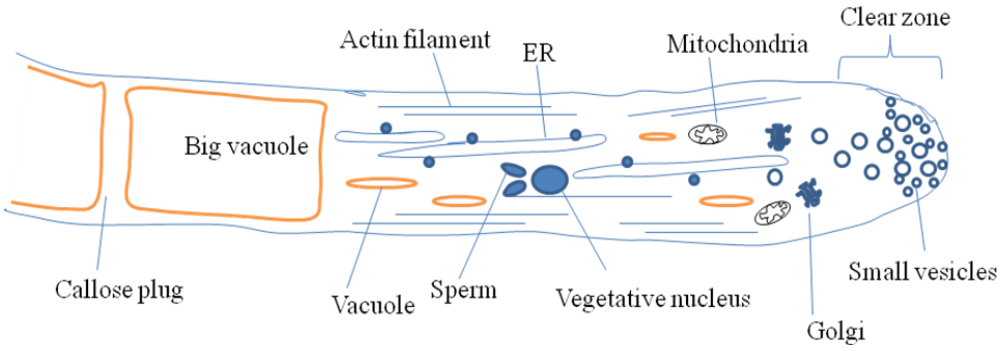


Fig. I-4. Growing pollen tube. (Modified from Franklin-Tong, 1999)

actin filaments and regulatory proteins, vesicle trafficking, and the Rac/Rop small GTPases.

3-1 Ion dynamics and regulations

Ion gradients and fluxes play a central role in the control of pollen tube growth (Holdaway-Clarke and Hepler, 2003). Very dynamic ion flux was found in the elongating pollen tube. A dramatic intracellular Ca^{2+} gradient in elongating tube has long been shown to be tightly coupled to cell elongation (Rathore et al., 1991). An acidic domain was also found at the extreme apex off growing pollen tube, even though the biological role in tip growth is controversial (Feijo et al., 1999). Oscillatory Ca^{2+} and pH levels at the tip accompany the oscillatory tube growth (Rathore et al., 1991; Lovy-Wheeler et al., 2006). K^+ is a central player in turgor regulation in the elongating pollen tube (Holdaway-Clarke and Hepler, 2003). Here I summarize the dynamics, regulation and role of these three ions in pollen tube growth.

3-1-1 Ca^{2+} & Ca^{2+}-dependent activities

Ca^{2+} is an essential ion for the pollen germination and tube growth (Brewbake and Kwack, 1963). Beside its role as nutrient, Ca^{2+} also act as guiding signal inducing tube chemotropic response in some species (Rosen, 1968). Optimal Ca^{2+} medium concentration for tube growth in vitro varies depending on species, like about 0.1 mM in *Tradescantia* pollen (Steer and Steer, 1989), but 10 mM in Arabidopsis (Fan et al., 2001). Growing pollen tube is characterized by a tip-focused Ca^{2+} gradient, which can be visualized by ratiometric ion imaging (Rathore et al., 1991). The Ca^{2+} concentration at the extreme tip reaches 3-10 μM, but drop sharply to 150-300 nM within 20 μm from the

apex (Holdaway-Clarke and Hepler, 2003). Disruption of the Ca^{2+} gradient stops tube growth, while inhibition of tube growth dissipates the Ca^{2+} gradient (Rathore et al., 1991; Pierson et al., 1994). More interestingly, the Ca^{2+} gradient at the tip oscillates in phase with tube growth (Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). Thus, tip-focused Ca^{2+} gradient is coupled to the tip growth. An interesting question is how the oscillatory tip-focused Ca^{2+} gradient is generated and maintained?

Isotope labeling and fluorescent probing showed that Ca^{2+} is taken into the tube tip (Jaffe et al., 1975). Using ion-selective electrode, Kuhtreiber and Jaffe (1990) detected a tip-directed Ca^{2+} influx, most possibly mediated by Ca^{2+} permeable channel (Sze et al., 2006). Indeed, presence of the Ca^{2+} channel blocker nifedipine resulted in disturbed tip Ca concentration and inhibited tube growth (Reiss and Herth, 1985). Several channel activities have been detected in pollen, include the voltage-gated channel in Agapanthus umbellatus pollen (Malho et al., 1995), stretch-activated channel in lily pollen (Dutta and Robinson, 2004) and hyperpolarization-activated channel in Arabidopsis (Wang et al., 2004) and lily pollen (Shang et al., 2005). Recently, a predicted cyclic nucleotide-gated channel, CNGC18, was cloned and shown to be localized to the pollen tube plasma membrane at the tip region. T-DNA insertion mutant of CNGC18 showed impaired pollen tube growth. Expression of the CNGC18 cDNA in E. coli cells resulted in more Ca^{2+} accumulation (Frietsch et al., 2007). Thus, CNGC18 could be a candidate channel for Ca^{2+} influx at the tube tip. Beside the extracellular Ca^{2+}, the internal stores of Ca^{2+}, for example, from the ER and small vesicles, could also contribute to the formation of the high Ca^{2+} concentration at the tip by releasing Ca^{2+} into the cytoplasm. So far, no intracellular Ca^{2+} channels have been cloned from pollen.

To maintain a tip-focused and oscillatory Ca^{2+} gradient, pollen tube must be able to remove cytoplasm Ca^{2+} in a spatially and temporally-controlled manner, but the mechanism is not clear. Expression of ACA9 in yeast strain that harbors native Ca^{2+} pump deletions complemented cell growth, suggesting ACA9 has Ca^{2+} pump activity. T-DNA insertion mutant pollen of ACA9 showed impaired fertility due to reduced tube growth and failed discharge. As ACA9 is localized to the pollen plasma membrane (Schiott et al., 2004), it could function in reducing Ca^{2+} concentration at the subapical region of pollen tube by pumping Ca^{2+} out of the cytoplasm, thus contribute to the Ca^{2+} gradient (Sze et al., 2006). Plant cells have other Ca^{2+} pumps and H^{+}/Ca^{2+} antiporters on endomembranes (Sze et al., 2000), suggesting these pumps and antiporters could play roles in creating the oscillatory tip-focused Ca^{2+} gradient, by translocating Ca^{2+} into endomembrane lumen in a spatially- and temporally-controlled manner (Sze et al., 2006). For example, pollen from the T-DNA insertion mutant of AtECA3, a Ca^{2+} pump on post-Golgi membranes, showed reduced tube growth in vitro (Li Xiyan, PhD dissertion ,2006). Overall, the cellular and molecular mechanism regulating Ca^{2+} dynamics in pollen is not clear.

In order for the Ca^{2+} to affect pollen tube growth, it needs to bind to and modify the activity of the target molecules. These targets include calmodulin (Snedden and Fromm, 2001) and Ca-dependent protein kinases (CDPKs) (Harmon et al., 2000). Calmodulin, CDPK and other Ca-binding proteins are highly expressed in pollen tube (Moutinho et al., 1998) (Honys & Twell 2004), but their role in pollen function is largely unknown. One study showed that the actin-binding protein villin needs the presence of both Ca^{2+} and calmodulin to unbundle actin filaments (Yokota et al., 2000), which is one

key component for pollen tube growth. In another study, it was shown that a calmodulin-binding protein is essential for pollen germination. Knockout mutant pollen showed severely reduced germination percentage (Golovkin and Reddy, 2003), which hints that Ca^{2+} and calmodulin are involved in this process. Based on other studies, it is thought that calmodulin binds to Ca^{2+} and regulates activities of other molecules, like pumps that in turn regulate cellular Ca^{2+} concentration (Snedden and Fromm, 2001).

Of 34 CDPKs in the Arabidopsis genome (Cheng et al., 2002), over ten are expressed in pollen (Qin et al., 2009). Estruch et al (1994) cloned the first pollen-specific CDPK in maize. Antisense pollen showed impaired germination and tube growth. One study in *Agapanthus umbellatus* pollen showed that CDPK activity shifted localization in response to reorientation signal, implying this CDPK plays an important role in tip growth (Moutinho et al., 1998). Two CDPKs from petunia were shown to be important for pollen tube growth. Pi CDPK1 was localized on pollen plasma membrane and played an important role in regulating polarity, while Pi CDPK1 was localized on punctuate endomembrane and regulate tube growth. But the mechanism is not clear (Yoon et al., 2006). Recently, another two CDPKs from Arabidopsis were shown to play overlapping but important role in tube growth. *Cpk17/34* double mutant pollen had reduced tube growth compared to wild type. Interestingly, by calculating fertilization efficiency of the mutant pollen tube, the authors proposed that mutant pollen failed to sense or change direction toward the ovules (Myers et al., 2009).

Considering the critical role of Ca^{2+} for pollen tube growth, there is still long way to go to elucidate mechanisms of how the dynamic gradient is regulated and how the ion gradient affects tip growth.

3-1-2 K^+

As an essential macronutrient for plant growth and development, K^+ is the most abundant cation in living plant cells. K^+ plays a number of fundamental roles inside cell, like osmoregulation, enzyme activation, electrical neutralization and membrane potential regulation. The turgor pressure of fast growing pollen tube must be maintained, as the cell has very dynamic vacuoles (Hicks et al., 2004) and streaming cytoplasm. The cytoplasm is pushed to the apical region of the tube by large vacuoles at the base, formation of which also require higher turgor pressure and so more K^+. Compared to the extensive studies of K^+ transport in other plant cells (Very and Sentenac, 2003), relatively little is known of how K^+ flux is regulated in pollen cell. Using patch-clamping, K^+ channel activities have been detected in pollen, including the hyperpolarization activated inward channel (Fan et al., 2001; Mouline et al., 2002), depolarization activated outward channel (Fan et al., 2003), stretch activated and spontaneous channel (Griessner and Obermeyer, 2003; Dutta and Robinson, 2004) and voltage-insensitive channel (Becker et al., 2004). Only two channels have been cloned so far. SPIK, one of nine Shaker channels in Arabidopsis genome, is expressed specifically in pollen. Expression of SPIK in mammalian COS cells showed hyperpolarization-activated K^+ channel activity. T-DNA insertion mutant pollen showed impaired tube growth, which suggest that SPIK mediated K^+ uptake is important for pollen tube elongation (Mouline et al., 2002).

Another K^+ channel, AtTPK4/KCO4, is a tandem-pore channel localized to the pollen plasma membrane. Expression of the TPK4 protein product in *Xenopus* oocytes showed voltage-independent K^+ channel activity. T-DNA insertion mutant pollen exhibited altered membrane electrical properties, although the mutant pollen showed normal germination and tube growth (Becker et al., 2004). Based on K^+ concentration in cytoplasm and pollen growth medium, it is also conceivable that K^+ could be taken into the cell via a H^+/K^+ symporter (Sze et al., 2006). Transcriptome data show many genes encoding predicted K transporters are expressed in mature pollen grain (Bock et al., 2006), and in growing pollen tubes (Qin et al., 2009), so more studies are needed to unveil their biological functions.

3-1-3 H^+, pH and its effectors

In addition to Ca^{2+}, pH emerges as another key regulator for pollen tube growth. Using the pH sensitive dye BCECF, Feijo et al (1999) showed that growing pollen tube has an acidic tip and an alkaline band at the base of the clear zone. Interestingly, this pH gradient was shown to be oscillatory in phase with tip growth, with an increase in pH at the apex preceding growth and a decrease in pH following growth (Lovy-Wheeler et al., 2006). Inhibition of tube growth dissipates the tip pH gradient and move the alkaline band toward the apex (Feijo et al., 1999), while dissipation of the pH gradient using buffer reduced or inhibited tube growth (Parton et al., 1997; Feijo et al., 1999). Little is known how the H^+ dynamics is regulated. H^+ exhibits a current loop around the apex region of the pollen tube. It is thought that H^+ influx occurs at the tip via channels and outflow at the base of the clear zone via plasma membrane H^+-ATPase (Hepler et al.,

2006). Plasma membrane H^+-ATPase are expressed in pollen grain and growing pollen tube (Honys and Twell, 2004; Robertson et al., 2004; Qin et al., 2009). Recently, a plasma membrane H^+-ATPase was found to be excluded from the apex and localized to the base of the clear zone (Certal et al., 2008), which may function to pump out the H^+ and thus contribute to the formation of the alkaline band. No H^+ channel has been cloned. Beside the PM, H^+ channel, H^+ pumps and H^+-coupled cotransporters on endomembranes could also contribute to the regulation of the oscillatory pH_{cyt} gradient (Sze et al., 2006). Dettmer et al. (2005) showed that vacuolar H^+-ATPase plays an essential role in Golgi organization and pollen development.

One of the effectors of H^+ in pollen tube is actin filament, an essential component for pollen tube growth (Vidali et al., 2001). Actin filaments depolymerized fast at pH 8.0 or above, but slowed down in neutral or slightly acidic pH (Andersland and Parthasarathy, 1993). The observation of this pH-dependent difference in actin dynamics might be attributed to the activity of ADF/cofilin, which severes actin filaments and is pH sensitive (Allwood et al., 2002; Cheung and Wu, 2008). Alkaline pH enhances the activity of ADF/cofilin. In fibroblast cells that lack the functional NHE1, a plasma membrane Na^+/H^+ exchanger regulating the efflux of H^+, less free barbed ends of actin filament was observed due to lower local cellular pH in the mutant cell (Frantz et al., 2008). ADF/cofilin is expressed in pollen. Green fluorescence protein (GFP)-tagged NtADF was localized on the base of the clear zone of the pollen tube, where axially oriented actin cables discontinue, actin mesh composed of short filaments was observed and streaming cytoplasm change direction (Chen et al., 2003). Colocalization of ADF with the fringe actin filament at the alkaline band may hint an interaction between these two. Bernstein

and Bamburg (2004) proposed that local pH modulation of ADF/Cofilin is the key regulator for actin remodeling and the defining of polarity site in animal cells.

Vesicle trafficking is dependent on pH (Caplan et al., 1987). Pollen tube elongation depends on active secretion of large amount of vesicle containing membrane and wall materials (Steer and Steer, 1989). Change in cellular pH alone can change vesicle trafficking between compartments and produce severely altered organellar morphology and movement in macrophage and fibroblast cells (Heuser, 1989). V-ATPase has been shown to be required for endocytic and secretory trafficking in Arabidopsis. Presence of V-ATPase inhibitor blocked the endocytic pathway from early endosome to tonoplast and secretory pathway from trans-Golgi network to plasma membrane (Dettmer et al, 2006). Loss of the V-ATPase function resulted in complete male and partial female gametophytic lethality possibly by blocking pollen tube growth (Dettmer et al, 2005). In yeast cells, mutation of the Na^+, K^+/H^+ cotransporter NHX1 resulted in abnormal prevacuolar structure and missorted vacuolar proteins (Bowers et al., 2000; Brett et al., 2005), suggesting a role of pH in protein sorting.

The AtCHXs are predicted to encode cation/proton exchangers based on sequence analysis (Sze et al., 2004). Several vegetative tissue-expressed CHXs have been studied. CHX20 was shown to be an endomembrane protein highly expressed in leaf guard cells and is important for light-induced stomata opening. Expression of CHX20 in KTA40-2 yeast mutant enhanced cell growth at alkaline pH and low external K^+, suggesting CHX20 play a role in mediating pH and K^+ homeostasis (Padmanaban et al., 2007). CHX13 protein was localized to plasma membrane and function to facilitate both yeast

and plant growth under low K^+ and acidic conditions (Zhao et al., 2008). Another CHX, CHX17 enhance yeast cell growth under low K^+ and alkaline pH conditions (Maresova and Sychrova, 2006). K^+ starvation and acidic conditions induced CHX17 gene expression in Arabidopsis, and knock-out mutant had reduced K^+ accumulate in the root under K^+ starvation (Cellier et al., 2004). Thus, all these studied CHXs showed function in regulating cellular K^+ and pH. Curiously, most of the 28 CHXs are highly expressed in pollen, though their biological function in pollen development and/or function is completely unknown.

3-2 Actin filaments and regulatory proteins

Actin filaments are essential for pollen tube growth (Vidali and Hepler, 2001). Growing pollen tube shows three major regions of different actin filaments arrangements and structures (Vidali and Hepler, 2001). The shank region contains longitudinal actin bundles that function with myosin to generate the cytoplasmic streaming to transport organelles and secretory vesicles toward the apex, the subapical region shows a ring-like actin structure that colocalize with the alkaline band and the ADF activity region (Kost et al., 1998; Vidali and Hepler, 2001; Vidali et al., 2001; Chen et al., 2002; Snowman et al., 2002; Lovy-Wheeler et al., 2005), and in the apical region very thin and short actin bundles were found (Fu et al., 2001). Actin depolymerizing drugs inhibited tube growth at concentrations lower than needed to interfere with the cytoplasm streaming (Gibbon et al., 1999; Vidali and Hepler, 2001), possibly by interfering the dynamics of the short actin bundles in the tip (Fu et al., 2001). More interestingly, the tip actin dynamics was

found to oscillate and lead growth (Fu et al., 2001). But how the dynamics of actin filament in pollen tube is regulated is poorly understood.

Actin binding proteins (ABPs) bind to and affect the activity of actin. Three types of ABPs have been identified in pollen, including profilin, ADF/cofilin and gelsolin family proteins (Yokota and Shimmen, 2006). Profilin is G-actin binding protein usually forms multigene family in plant species. Arabidopsis genome has five profilin isoforms, among which two are specifically expressed in pollen (Kandasamy et al., 2002). Profilin distributes uniformly inside pollen tube (Vidali and Hepler, 1997; Kandasamy et al., 2002). Sharing similar activity of their animal counterparts, plant profilins bind to G-actin and facilitate actin polymerizing at the free plus end at high concentration of G-actin. Conversely, binding of profilin sequesters G-actin from spontaneously neucleation and prevent actin polymerization (Yokota and Shimmen, 2006). Profilin activity is Ca^{2+}-dependent, higher Ca^{2+} concentration enhances profilin binding and sequestering G-actin from being polymerized.

ADF/cofilin is another ABP that binds and modulate actin dynamics in all known eukaryotic cells. ADF/cofilin dissociates actin subunit from the minus end and increases the rate of actin filament treadmilling. Furthermore, ADF/cofilin severs actin filaments and creates more ends for polymerization (Maciver and Hussey, 2002) and defines direction for polar cell motility (Ghosh et al., 2004). Arabidopsis genome contains at least nine ADF/cofilins (Dong et al., 2001), among which 7 are expressed in pollen (Honys and Twell, 2004). ADF/cofilin activity is pH sensitive as discussed above. ADF/cofilin activity is also regulated by phosphorylation and phosphoinositides. Phosphorylation of

the conserved Ser6 in the N terminal by the Ca^{2+}-dependent protein kinase reduced the ADF/cofilin actin-binding and depolymerizing activity (Smertenko et al., 1998; Allwood et al., 2001). Phosphoinositides bind and inhibit ADF/cofilin activity (Allwood et al., 2002), so phosphoinositides may help to localize ADF/cofilin activity and thus the actin dynamics in growing pollen tube.

Gelsolin family proteins are the third class of actin regulators. Activity of these proteins is Ca^{2+} dependent (McGough et al., 2003). High level of Ca^{2+} enhances gelsolin binding and severing actin filaments (Fan et al., 2004; Huang et al., 2004). Gelsolin severs actin filaments and bind to the plus end acting as a capping protein, until phosphoinositides, like PtdIns(4,5)P_2 come to bind to the actin and dissociate it. In Arabidopsis genome, no gelsolin gene was found (Yokota and Shimmen, 2006), but other members of the Gelsolin family proteins, like villin, was expressed (Huang et al., 2005). Overall, little is known about this family in growing pollen tube.

3-3 Vesicle trafficking

Growing pollen tube has highly dynamic endomembrane systems, including the vacuole in the cytoplasm below the subapical region (Hicks et al., 2004; Lovy-Wheeler et al., 2007), the ER (Lovy-Wheeler et al., 2007) and small vesicle in the apical region (Steer and Steer, 1989; de Graaf et al., 2005). Disrupting membrane trafficking along the secretory pathway reduced the population of small vesicles at the tip region and inhibited pollen tube growth, suggesting the essential role vesicle trafficking plays for the astonishing rapid pollen growth (de Graaf et al., 2005). These membrane movement and vesicle trafficking are actin dependent, alteration of the actin dynamics by actin drugs

(Cheung et al., 2002; Lovy-Wheeler et al., 2007) or modifying ADF expression (de Graaf et al., 2005) disturbed the membrane movement and inhibited tube growth (Lovy-Wheeler et al., 2007). Besides providing new membrane and wall materials to the growing site, the small secretory vesicles at the apical region may also play an important role in pollen-female cell interaction by delivering receptors to the plasma membrane (Muschietti et al., 1998).

Quantity of membrane delivered by exocytosis clearly exceeds requirement for the cell growth rate, indicating existence of coupled endocytosis to recycle the over-delivered materials (Steer and Steer, 1989) . Evidence from early experiment suggests existence of endocytosis pathway in pollen tube. These include high density of clathrin-coated pits in pollen tip (Derksen et al., 1995), clathrin heavy chains localized to the tip region of pollen tube (Blackbourn and Jackson, 1996) and detection of female cell-secreted molecules inside pollen tube (Lind et al., 1996; Luu et al., 2000). By labeling plasma membrane with the membrane impermeable dye FM4-64, Parton et al (Parton et al., 2001) observed directly the active endocytosis in the apical region of growing pollen tube. Thus, the pollen tube apical region is packed with active exocytsis as well as endocytosis vesicles. Possibly, by this combined membrane trafficking, pollen regulates not only the growth speed by balancing the amount of membrane and wall materials added to the growing site, but also interactions with the surrounding female cells, as shown by the SI response in *Solanum chacoense* (Luu et al., 2000). Further, these vesicle trafficking may regulate pollen-female cell cross-talking by modifying the population of receptor molecules on the plasma membrane, as seen in other plant cells in response to changing boron levels (Takano et al., 2005) or biotic environment (Robatzek et al., 2006).

Homologs of molecules that regulate vesicle trafficking in eukaryote cells are found in the Arabidopsis genome (Arabidopsis Genome Initiative, 2000; Jurgens, 2004). In pollen, several regulators have been functionally identified, including the Rab small GTPase and exocyst complex. The Rab small GTPase proteins are key regulators of vesicle trafficking (Molendijk et al., 2004). Arabidopsis has 57 Rab GTPase proteins that sort into eight groups based on phylogenic analysis (Vernoud et al., 2003), though only a few have been functionally identified. NtRab2, a homolog of the Arabidopsis RabB family in tobacco pollen, was shown to be a Golgi localized protein that regulates vesicle trafficking between ER and Golgi. Modulation of the activity of NtRab2 by either dominant negative (DN) or overexpression disturbed protein targeting and inhibited tube growth (Cheung et al., 2002). Another tobacco Rab, Rab11, a homolog of the Arabidopsis RabA1 subfamily, was localized mainly to the small secretory vesicles in the apical clear zone of pollen tube. DN or CA (constitutive active) Rab11 transformed pollen tube failed to deliver cell wall and secreted proteins and tube showed slower and kinky growth. More interestingly, these transformed pollen tubes also showed disturbed endocytosis membrane recycling (de Graaf et al., 2005). Thus, Rab11 play an important role in regulating pollen tube tip growth by mediating both exocytosis and endocytosis to produce polar delivered vesicles to the growing site. The first functionally identified pollen Rab in Arabidopsis, RabA4d, was shown to be localized to the membrane compartments in the tip that exclude the endocytic membranes, which is different with the NtRab11 (Szumlanski and Nielsen, 2009). T-DNA insertion RabA4d mutant pollen displayed reduced growth and lost polarity, which suggest that RabA4d GTPase play an important role in regulating rapid tip growth, but the mechanism is not clear. Interestingly,

RabA4d was found to bind to PI4Kβ1, a phosphatidylinositol 4-OH kinase that in turn binds to a Ca^{2+} sensor, AtCBL1, thus sensibly the tip-focused Ca^{2+} gradient and tip vesicle trafficking was connected via lipase and the Rab small GTPase. Further, this PI4Kβ1also bind to RabA4b, another small GTPase that was localized to the endomembranes in the tip region of growing root hair, another plant cell that shows tip growth (Preuss et al., 2004; Preuss et al., 2006). These results suggest the shared cellular mechanism controlling polar cell growth in Arabidopsis.

Overall, how the Rab small GTPase proteins are involved in the pollen tube growth is poorly understood. More questions need to be answered, like how many of the 57 Rabs are involved in the pollen tube growth? What are the specific roles of each in tube growth? What membrane compartment does each Rab regulates? How are these Rabs coordinatedly regulated?

The exocyst plays an essential role in trafficking by tethering approaching vesicle to the exocytic site on the target membrane in yeast and animal cells (Lipschutz and Mostov, 2002), and possibly in pollen tubes as well. The exocyst is an octameric protein complex (Munson and Novick, 2006). Homologs of all the eight subunits were found in the Arabidopsis genome (Analysis of the genome sequence of the flowering plant Arabidopsis thaliana, 2000), among which only a few have been functionally identified in tip growth. Genetic data showed that SEC3 and EXO70A1 play important roles in root hair tip growth, as *roothairless1 (rth1)/sec3* (Wen et al., 2005) and *exo70A1* (Synek et al., 2006) T-DNA insertion mutants showed failed root hair initiation and/or elongation. AtSEC8 was shown to be critical for pollen germination, as T-DNA mutants failed to

grow a tube when put onto the stigma (Cole et al., 2005). These proteins are thought to be subunits of a functional exocyst complex. By regulating exocytosis they may play important roles in initiating and sustaining tip growth in pollen and root hair. Interestingly, SEC3A was found to interact via an effector with ROPs (Lavy et al., 2007), the "master regulators" for polar cell growth in plants (Lee and Yang, 2008).

3-4 Rac/Rop small GTPases

The Rho-related plant-specific Rac/ROP GTPases are the "signaling hubs" in pollen (Nibau et al., 2006; Lee and Yang, 2008). These small GTPases exist either in the GTP-binding active or GDP-binding inactive state, and function as molecular switch in regulating signaling transduction in many cellular processes. Arabidopsis genome contains over 10 ROP genes, among which four are highly expressed in mature pollen and growing pollen tube (Honys and Twell, 2004; Qin et al., 2009). Extensive studies from Zhenbiao Yang's group showed that Rop1 GTPase function as "master regulator" during pollen tube growth. ROP1 was localized to the plasma membrane apex of the pollen tube (Lin et al., 1996). This polar localization play essential role in regulating polar tip growth, as ectopic expression of ROP1 in ROP1 constitutive active pollen resulted in tube depolarization and bulged tube growth (Fu et al., 2001). Activities of Rop GTPase oscillate in phase and leads pollen tube tip growth (Hwang et al., 2005). Elegant studies showed that ROP1 regulates pollen tube growth through two counteracting pathways (Gu et al., 2005). The RIC4 mediated pathway polymerizes apical actin filament and is important for secretory vesicle accumulation to the tip of pollen tube, while the RIC3 mediated pathway depolymerizes apical actin filament in a Ca^{2+}-

dependent manner and is important for vesicle fusion to the plasma membrane (Lee et al., 2008). Thus, by regulating apical actin dynamics, ROP1 mediates vesicle delivery and fusion at the apical plasma membrane and oscillatory tube growth (Lee and Yang, 2008). But the mechanism of how ROP1 activity is located at the apex of pollen tube is not clear.

A recent study in root hair growth showed that the ROP1 effector ICR1 may play a role in recruiting ROP1 specifically to the apical plasma membrane (Lavy et al., 2007). Interestingly, ICR1 has a similar role in pollen (Lee and Yang, 2008), though the molecular mechanism is not clear. ICR1 was localized to the apical plasma membrane of pollen tube, and overexpresson of this protein enhanced ROP1 recruitment to the apical PM. GDI (guanine nucleotide dissociation inhibitor) and GAP (GTPase activating protein) are two negative regulators of Rop GTPase and have been shown to play an important role in restricting ROP activity to the apical region of pollen tube. By sequestering ROP in the cytoplasm from the flanking plasma membrane, NtRhoGDI2, may function to localize tobacco ROP homolog NtRac5 to the apex (Klahre et al., 2006). The same research group found that the GFP-tagged NtRhoGAP1, a tobacco homolog of Arabidopsis GAP, was preferentially localized to the flanking region of pollen tube. NtRhoGAP1 showed GTPase activating activity and bound to NtRac. Mutation of the GAP activity of NtRhoAGP1 resulted in bulged tube growth resembling those ectopic NtRac5 expression, while expression of a NtRac5 competitor protein that binds to NtRhoAGP1 alleviate NtRhoAGP1 overexpression-caused growth inhibition (Klahre and Kost, 2006). These results suggest that GAP function in limiting and maintaining ROP activity at the apical region.

Recent results suggest that besides regulating pollen tube tip growth, ROP GTPase may also be involved in RLK (receptor-like kinase)-mediated interaction between the pollen tube and the surrounding female cells. Two RLKs were identified in tomato pollen tube (Muschietti et al., 1998). Molecules from both pollen and female cells bind these RLKs and these associations are important for stimulating pollen germination and tube growth (Tang et al., 2002; Tang et al., 2004; Zhang et al., 2008). Interestingly, a pollen-specific RopGEF was found to bind tomato PRK2 in yeast two hybrid and in vitro binding assays (Kaothien et al., 2005). Further study showed that Arabidopsis PRK2 interacts with and may regulate RopGEF12 activity by phosphorylation and by suppressing the autoinhibition of the C tail of the RopGEF12 (Zhang and McCormick, 2007). Thus, identification of this RLK-mediated signaling pathway indicates that ROP-GTPase may play an important role in sensing or transducing external guiding signals from the female cells and the following tube reorientation.

4. Pollen-pistil interaction

One fundamental question is how does the pollen tube find an ovule after penetrating the transmitting tissues of the pistil? Studies are revealing that female cells along the pollen tube pathway provide guiding signals and pollen tubes very likely sense, interpret and respond to these cues (Fig. I-3.). Research in the last decade showed clearly the existence of pollen tube guidance before successful fertilization, though the understanding of the molecular bases of the process is at its infancy.

4-1. Female guiding cells

Both sporophytic and gametophytic female cells have been shown to be involved in pollen tube guidance. Stigma and stylar cells play a role to capacitate pollen tube to respond to late stage guiding signals, as pollen germinated completely in vitro failed to target ovule in the semi in vivo experiment. In contrast, pollen tubes that went through the stigma and stylar cells are directed towards excised ovules in either Torenia (Higashiyama et al., 1998) or Arabidopsis (Palanivelu and Preuss, 2006). Normal development of the ovary transmitting tract cells are required for both tube elongation and guidance, in the Arabidopsis *ntt* (NO TRANSMITTING TRACT) mutant, pollen tube growth was retarded and diverted to the side of the ovary, instead of going downward to the far end of the pistil (Crawford et al., 2007). Thus many female sporophytic cells contribute to mediating pollen tube guidance.

Genetic data showed that the ovule and the female gametophyte (FG) are essential for tube guidance at the late stage, the gametophytic guidance. In mutant plants that have disrupted or delayed ovule and/or FG development, tube guidance failed (Hulskamp et al., 1995; Ray et al., 1997; Shimizu and Okada, 2000) . Using micro-ablation, Higashiyama et al (2001) showed that Torenia synergid cells play an essential role for gametophytic tube guidance. Consistently, in the Arabidopsis *myb98* mutant, in which synergid cell development was specifically affected, pollen tube guidance was disrupted (Kasahara et al., 2005). Possibly, synergid cell attracts pollen tube by secreting diffusible molecules that are species-specific (Higashiyama et al., 1998; Palanivelu and Preuss, 2006). Interestingly, the central cell was also shown to play a role in tube guidance (Chen et al.,

2007), perhaps due to effects on the neighboring synergid cells. Multiple stage-specific guiding signals are proposed to mediate pollen tube guidance (Johnson and Lord, 2006), maybe by secreting different guiding signals by different female cells along the tube growth pathway.

4-2 Female guiding signals

Several molecules appear to serve as guiding signals to promote and/or change direction of tube growth, including lipids from the tobacco stigma cells (Wolters-Arts et al., 1998), chemocyanin in the lily stigma (Kim et al., 2003), arabinogalactan proteins in tobacco transmitting tissue (Cheung et al., 1995; Wu et al., 1995) and γ-aminobutyric acid along the pollen tube growth pathway in Arabidopsis (Palanivelu et al., 2003), and ZmEA1, a small secreted protein from the maize egg apparatus (Marton et al., 2005). But direct genetic and molecular evidence are needed to verify these as true pollen tube attractants (Higashiyama and Hamamura, 2008). Recently, two cysteine-rich, plant defensin-like proteins were identified as secreted products from synergid cells in Torenia. In vitro assay showed that these two protein attract pollen tube and cause tube reorientation when applied on pollen growth medium (Okuda et al., 2009). The FERONIA, a synergid cell-specific receptor-like kinase, plays an essential role in pollen tube reception (Escobar-Restrepo et al., 2007), mutation of this gene caused continued tube growth and multiple tube entries inside the receptive synergid cell (Huck et al., 2003; Rotman et al., 2003; Escobar-Restrepo et al., 2007). These molecules illustrate the diversity of signals for tube growth and guidance.

4-3 Pollen tube response

Even though guiding signals from the female cells are being identified, how the pollen tube senses and responds to these various guiding signals is poorly understood. A pollen aminotransferase, POP2, was suggested to play a role in pollen tube guidance possibly by creating a GABA gradient along the pollen tube pathway toward ovule (Palanivelu et al., 2003). Surprisingly, a sperm-specific protein, HAP2, was shown to mediate pollen tube guidance, suggesting that the sperm cell is not a passive passenger, but also appears to participate in the directionality of the growing tube (von Besser et al., 2006), but the mechanism is not known. Pollen receptor-like kinases (RLKs) could be plausible candidates for sensing external signals. The Arabidopsis genome contains more than 600 RLKs (Shiu and Bleecker, 2001), among which more than 20 are expressed in pollen (Honys and Twell, 2004). Two pollen specific RLKs, LePRK1 and LePRK2, were shown to be on the plasma membrane of tomato pollen (Muschietti et al., 1998). Binding of ligands from both pollen and the female cells enhanced pollen germination or tube growth (Tang et al., 2002; Guyon et al., 2004; Zhang et al., 2008). Interestingly, the cytoplasmic domain of the LePRK2 interacts with one member of the RopGEF family (Guanine nucleotide exchange factors) that control the activity of the pollen Rop (Rac-like) small GTPase (Kaothien et al., 2005), which are in turn a key molecule for signal integration in controlling pollen tube growth (Nibau et al., 2006; Yang and Fu, 2007). Interestingly, ROP1 GTPase was shown to bind via an effector with SEC3 (Lavy et al., 2007), a component of the exocyst complex for tethering exocytic vesicles to the target site on the PM for localized exocytosis (Cole and Fowler, 2006). All these characterized molecules could be part of a signal apparatus that sense external signal (s) and transduce

the signal (s) into the pollen and cause changes in tube growth in terms of rate and direction. More work need to be done to illuminate this type of signaling pathway and integrate this pathway with other components that control pollen tube growth.

5. Summary /conclusion

Although considerable information has emerged regarding the cellular and molecular mechanisms regulating *in vitro* pollen tip growth, much less is known about how the pollen tube communicates *in vivo* with the female cells and then respond to their cues to alter tube growth and guidance. The transcriptome of developing pollen grain and the pollen tube are revealing many genes that are preferentially and specifically expressed in pollen, including predicted pollen receptors, signaling proteins and ion transporters. Curiously, one transport gene family stands out in particular as 14-18 members of this cation/proton exchange (CHX) family are expressed in pollen.

B. STATEMENT OF THE PROBLEM & RESEARCH GOALS

Many members of the AtCHX gene family are expressed in pollen, although their role in plant reproduction is not known. The objective of this work was to test the role of CHX21 and CHX23, the two closely-related homologs, in pollen development and function. I chose these two because they are the only members that form a branch on the CHX family tree, not like other CHXs that with multiple members aggregate in a group. Specific objectives were:

1. Determine whether single and double mutants of chx21 and chx23 were defective in pollen development, germination, tube growth or fertilization. I showed that *chx21* or *chx23* single mutant pollen, is fully functional in terms of germination, tube growth and fertilization; and 2) *chx21chx23* double mutant pollen functions normally in germination and tube growth, but lose its ability to direct its growth toward the ovule at the funiculus and micropylar stages (Chapter II).

2. Understand the molecular function of CHX23 by determining its membrane localization in pollen and its activity. The CHX23-GFP protein was localized to the ER, either when it was transiently expressed in tobacco pollen, or stably expressed in Arabidopsis pollen under its native promoter. Expression of CHX23 in E. coli strains defective in K^+ uptake systems support the idea that CHX23 has a transport role that mediates cellular K^+ and pH homeostasis (Chapter III).

II. ANALYSES POLLEN DEVELOPMENT, GERMINATION, TUBE GROWTH AND FERTILIZATION IN *CHX21* AND *CHX23* MUTANT

1. ABSTRACT

CHXs are predicated cation/proton exchangers many of which are highly expressed in pollen, though the biological function of these genes are unknown. A reverse genetic approach was used to determine the function of two genes, CHX21 and CHX23, in Arabidopsis pollen. T-DNA insertional mutants of CHX21 and CHX23 were identified and analyzed. Single mutant pollen showed *in vitro* germination and tube growth similar to that of wild type pollen. The mutant segregation ratio in the descendants of selfed single heterozygous plants was normal. However, mutant transmission mediated by pollen was specifically blocked when both genes are mutated. When wild-type pistil was pollinated with a limited number of pollen grains from *chx21-2/+ chx23-4/chx23-4* plants, seed set was reduced to about one thirds of that from *chx23-4* single mutant plants, suggesting that the *chx21chx23* double mutant pollen is infertile. The development and viability of *chx21chx23* pollen were not affected as visualized by DAPI and Alexander staining, respectively. I then tested in vivo pollen tube growth of chx mutants tracked by the GUS reporter. Single *chx21* pollen tube grew inside the transmitting tissues, turned towards the ovule and discharged its content into the embryo sac. However, the tube of the *chx21chx23* double mutant pollen remained inside the transmitting tissue and failed to target the ovule. In semi-*in vivo* assays, wild-type pollen tubes emerging from an excised style showed 91.5% successful targeting in 235 ovules, while only 11.5% of 183 ovules were targeted by *chx21chx23* pollen tubes. These results

suggest that *chx21chx23* double mutant pollen was incompetent to target ovules possibly due to failure to respond to ovule guidance cues. Together, these studies showed that CHX21 and CHX23 are essential for male fertility, and perform key role (s) in pollen tube targeting to the ovule.

2. INTRODUCTION

In flowering plants, sperm cells develop inside the male organ anther, while egg and central cells form and are deeply buried inside the female organ pistil. For these spatially separated sex cells to meet and fuse, plants use the vegetative cell of the male gametophyte, as a delivery tool. Mature pollen grain has a large vegetative cell with two sperm cells inside (Fig. I-1) (McCormick, 2004). After landing on appropriate stigma surface, the pollen vegetative cell germinates and protrudes to form a tube. The tube penetrates the pistil tissues and grows towards the ovule to deliver the two sperm cells to the egg and the central cell. So, reproduction of flowering plants depends on the function of the pollen tube. But the mechanism underlying the pollen tube directional growth is poorly understood.

2-1. Fast & competitive pollen tube growth

In Arabidopsis, each flower produce tens of hundreds of pollen grains, though each pistil contains only 40-50 ovules. Pollen out-number ovule resulting in intense competition among pollen, thus tube growth speed is an important determinant of pollen competitive ability to reach the next unfertilized ovule. The distance traveled by the pollen tube can be enormous compared to the diameter of the pollen tube itself and the tube growth speed is astonishing. In Arabidopsis, the growing pollen tube has a diameter of about 5 μm, while the distance between the stigma cells to the ovules at the bottom end of the pistil can be up to 3 mm (Fig. I-3). Within about 10 h, pollen tube reaches the ovules at the longest distance (Faure et al., 2002), showing a growth speed of about 83 nm/second. In tobacco, pollen tube growing in vivo reaches ovule at a distance of 4.5 cm

in about 30 hours, achieving an astonishing growth rate of about 400 nm/second (Cheung et al., 1995), faster than the neuron cell growth rate in Xenopus dorsal Rohon-Beard neurons, which is about 15 nm/second (Gomez and Spitzer, 1999). More interestingly, pollen tube shows oscillatory growth pattern (Hepler et al., 2001), but the underlying mechanism is not clear.

2-2. Subcellular components associated with tip growth

Several ion transporters have been shown to be important for fast tube growth and pollen competition. SPIK, a K^+ channel specifically expressed in pollen, was shown to be important for tube growth. T-DNA disruption of the SPIK gene impaired pollen tube growth *in vitro* and reduced gene transmission by the mutant allele. By regulating K^+ uptake and pollen osmotic potential, SPIK plays critical role for pollen tube growth and competition ability (Mouline et al., 2002). ACA9, an auto inhibited Ca^{2+} ATPase (Schiott et al., 2004), and CNGC18, a predicted cyclic nucleotide-gated channel (Frietsch et al., 2007), both are important for pollen tube growth by regulating cellular Ca^{2+} dynamics. T-DNA insertion in both genes reduced pollen tube growth and male fertility. As Ca^{2+} effectors, CDPKs were found to be important tube growth regulators. For example, when two Arabidopsis CDPK homologs, CPK17 & 34 were knocked out, pollen tube growth was impaired (Myers et al., 2009). Many other transporters are expressed specifically or preferentially in pollen, though their functions in pollen tube growth are not known (Sze et al., 2004; Bock et al 2006).

In addition, other molecules play a role in tube growth by regulating polar tip growth (Hepler et al., 2001). From extensive in vitro studies, several key cellular and

molecular components for the sustained tip growth have been identified. This include ions and the regulating transporters (Holdaway-Clarke and Hepler, 2003; Sze et al., 2006), actin and actin regulatory proteins (Vidali et al., 2001; Chen et al., 2002; Chen et al., 2003), vesicle trafficking and the regulator Rab small GTPases (Cheung et al., 2003; de Graaf et al., 2005; Szumlanski and Nielsen, 2009), and the Rop/Rac small GTPases (Nibau et al., 2006; Lee and Yang, 2008). These components are coordinated possibly by the Rop, and sustain and restrict cell growth to the tip (Fig. I-2) (Cheung and Wu, 2008), but the underlying cellular and molecular mechanisms are still illusive.

2-3. Guidance of pollen tube growth by female cues

After the pollen tube goes through long distance and penetrate multiple female cellular layers, the tube is competent to change direction and target an unfertilized ovule with high accuracy (Fig. I-3). Pollen tube growth and guidance can be divided into six phases: stigma penetration, growth in the transmitting tract, transmitting tissue exit, funicular guidance, micropylar guidance and pollen tube reception (Johnson and Lord, 2006). Different female cells, both sporophytic and gametophytic, have been implicated to play important role in pollen tube guidance. These include stigma cells (Wolters-Arts et al., 1998; Kim et al., 2003), stylar and ovary transmitting tissue cells (Palanivelu et al., 2003; Crawford et al., 2007), synergid cells (Higashiyama et al., 2001) and the central cell (Chen et al., 2007). Candidate molecules secreted from the female cells, include lipids from the wet stigma of Petunia (Wolters-Arts et al., 1998), arabinogalactan protein TTS from tobacco transmitting tissue (Cheung et al., 1995), γ-aminobutyric acid (Palanivelu et al., 2003), chemocyanin (Kim et al., 2003), small secreted protein EA1

from maize egg apparatus (Marton et al., 2005) and the defensin-like Lure proteins secreted from synergid cells (Okuda et al., 2009). 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.

2-4. Pollen perception and response to guidance cues are not understood

Much less is known about how pollen senses and responds to the various chemical signals *in vivo*. Forward genetic studies of male gametophyte mutants have so far yielded mutants with defects in development, or tube growth, but very few mutants impaired specifically in guidance (Johnson et al. 2004; Twell et al 2006). Several membrane-bound RLK kinases are expressed in Arabidopsis pollen (Honys and Twell, 2004), though their roles are just beginning to be characterized. A tomato PRK2 was shown to bind a pollen-specific Lat52 protein, and this Cys-rich protein is essential for pollen hydration and tube growth (Tang et al., 2002). PRK2 also binds to a Cys-rich secreted protein from the pistil LeSTIG, which stimulates pollen tube growth in vitro (Tang et al., 2004). These studies would suggest that PRK2 is involved in pollen-pistil interactions, though it is unknown whether PRK2, its homologs or other subfamilies of receptor like-kinases perceive and then transduce guidance cues. HAP2/GCS1, a sperm-specific membrane protein, has been shown to be important for gamete fusion, though it

also plays a role in tube guidance (von Besser et al., 2006). However, the molecular mechanism is unclear.

In this study, I used a reverse genetic approach to identify two cation/proton exchangers, CHX21 & CHX23, that are functionally redundant. Curiously, pollen carrying mutation in both CHX21 & CHX23 showed normal development, germination and tube growth in the transmitting tract, however their tubes failed to reorient and grow toward the ovule. Using a semi-*in vivo* assay, I further demonstrated that these CHXs are critical in pollen tube targeting to isolated ovules. Thus, CHX21 & CHX23 are key players for pollen tube guidance at the funicular and micropylar stages.

3. MATERIALS AND METHODS

3-1. Plant materials

Wild-type Arabidopsis thaliana (ecotype Col-0), mutants, and transgenic plants (Tab. II-1) were grown in Miracle-Gro potting soil (Scotts) under well-controlled environmental conditions at 21 ºC, 150 µE m^{-2} s^{-1}illumination for a 16 h photoperiod at 55% humidity.

Seedlings sterilized and grown on 1/2 MS plates: Arabidopsis seeds were surface sterilized, vernalized at 4ºC for 2 days, and germinated and grown on ½ MS plants under the same growth conditions as mentioned above.

3-2. Genotyping and segregation analysis

Genomic DNA was isolated from 2-week old seedlings. Gene-specific and T-DNA primers were used to identify heterozygous and homozygous mutants by PCR (Table II-2 for primers used). Segregation analyses of SALK mutants (*chx21-2* and *chx23-4*) or of out-crosses were determined by PCR-based genotyping.

SAIL mutant carries a T-DNA with a $Basta^R$ cassette (McElver et al. 2002), so segregation of self-fertilized *chx21-s1*$^{+/-}$ plants was scored as Basta resistance. Sterilized seeds were germinated on 0.5 X Murashige and Skoog plates containing Basta (50 mg/L) for 2 weeks. To determine the genotype of *chx21-2*$^{+/-}$*chx23-4*$^{-/-}$ mutant transformed with a binary vector containing the wild-type gCHX23-GFP and $hygromycin^R$, T_1 transformants were selected on plates containing 30 µg/ml hygromycin, and then subjected to PCR-

Table II-1. Arabidopsis plant lines used in this study.

Wt name	Mutant name	Identification no/ description	Source
	chx21-2	Salk_085865	Alonso et al. 2003 Science 301: 653-657
	chx21-s1	Sail_162_A07	Sessions et al, 2002 Plant Cell 12: 2985-2994
	chx23-4	SALK_035909	Alonso et al. 2003 Science 301: 653-657
Wt		Wt, ecotype Col-0	
Wt_i	*Imp-1 (aos)*	Enhancer trap mutant At5G42650	From Thomas Jack (Dartmouth).Dr. Z. Liu
Wt_{GUS}		pLat52 :: GUS	Dr. Ravi Palanevilu (Univ Arizona)

Table II-2. Primers used in this study. Enzyme site is shown in **Bold**.

Purpose	Primers	Primer Sequences
cDNA Cloning	CHX23Cf	5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC** TCCGACCAGCCATCGTAGGA-3'
	CHX23Cr	5'-**GGGGACCACTTTGTACAAGAAAGCTGGGTC** ATCCTCTTCATCATCTTCATCTTCG-3'
pPAB404-CHX23 cloning	AtCHX23-BglII-SE	5'-**GAGAGATCT**ATGTCTTCCGGAGCCCC-3'
	AtCHX23-XhoI-AN	5'-**ATTCTCGAG**TTACCTATGAATTCCATATTGATG-3'
CHX23 genomic DNA cloning	CHX23GCf	5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC** GCTACACTCCTAGATCAGAGTAAACAA-3'
	CHX23GCr	5'-**GGGGACCACTTTGTACAAGAAAGCTGGGTC** CCTATGAATTCCATATTGATGATCC-3'
Lat52 promoter cloning	Lat52 Pf	5'-**CGAGCTC** ACTCGACTCAGAAGGTATTGAGGA-3'
	Lat52 Pr	5'-**GGACTAGT** TTTAAATTGGAATTTTTTTTTTTGGTG-3'
chx21-2 RT-PCR	X21RT-F3	5'-AGAGCTGATATTGGTTACATGAAC-3'
	X21RT-R3	5'-GATTCAAACCGGACAGTCGT-3'
	X21RT-F4	5'-GGAGAGTCAGAGCTTTGTGTATTGA-3'
	X21RT-R4	5'-TGAGGTCACAGGTGTTTCTACTTGA-3'
	X21RT-F5	5'-TGACTTATGTAGAGAAAGTGGTTA-3'
	X21RT-R5	5'-TCATCTGCGCATACTGTACTGA-3'
chx21-s1 RT-PCR	X21RT-F1	5'-CTGAATCTATTGATGCAAGTTCGT-3'
	X21RT-R1	5'- GAAAGGAGGAAGACATAGAGGCTTA-3'
	X21RT-F2	5'-CAACTGTCTGTCGCTAATCTTACCT-3'
	X21RT-R2	5'-AGAATCCAAGTGCACAAATCAGTAAC-3'
	X21RT-F4	5'-GGAGAGTCAGAGCTTTGTGTATTGA-3'
	X21RT-R4	5'-TGAGGTCACAGGTGTTTCTACTTGA-3'
chx23-4 RT-PCR	X23RT-F1	5'-TAGCCTCTTCCCTTCCGTTC-3'
	X23RT-R1	5'-CACAAATGATGTAGAAGAGAGGCA-3'
	X23RT-F2	5'-TGGTCTGTCGCGCTAGCCT-3'
	X23RT-R2	5'-ACTAGAAGCGCGATCGTCATATGAGTAT-3'
	X23RT-F3	5'-CGTGGTGAATCAATGAAAAGAGAAGT-3'
	X23RT-R3	5'-ACATAGACTTCATAAATGGCTCATGA-3'
Actin11 RT-PCR	Actin-S	5'-ATGGCAGATGGTGAAGACATTCAG-3'
	Actin-AS	5'-GAAGCACTTCCTGTGGACTATTGA-3'
chx21-2	X21-2-LP	5'-TTTTTGCTATCCACCTAGTCGAGCTAAC-3'

PCR	X21-2-RP	5’-CGGTTTGTCTGTTAGCAGAAGAGTATTGT-3’
	LBa1	5’-TGGTTCACGTAGTGGGCCATCG-3’
chx21-s1 PCR	X21-S1-LP	5’-GTTTAAATCTATTAAGGAAAATCCCCC-3’
	X21-S1-RP	5’-AGAATCCAAGTGCACAAATCAGTAAC-3’
	pCSA110-LB3	5’-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3’
chx23-4 PCR	X23-4-RP	5’-ATCAGGTAAAGATTCCTACAATTAGTTCA-3’
	X23-4-LP	5’-ACTAGAAGCGCGATCGTCATATGAGTAT-3’
	LBa1	5’-TGGTTCACGTAGTGGGCCATCG-3’
CHX23-GFP trans-gene	X23G-F	5’-GATCCTTATGCCTCTCTTCTACATC-3’
	X23G-R	5’-CTAATTCAACAAGAATTGGGACAAC-3’
CHX23 cDNA Sequencing	X23CS1	5’-TAGCCTCTTCCCTTCCGTTC-3’
	X23CS2	5’-CACAAATGATGTAGAAGAGAGGCA-3’
	X23CS3	5’-ACTAGAAGCGCGATCGTCATATGAGTAT-3’
	X23CS4	5’-TCTGCTCTGACTCTGTCCGA-3’
	X23CS5	5’-GGATGAGATTAGAGCTTCGC-3’
	X23CS6	5’-ACATAGACTTCATAAATGGCTCATGA-3’
g CHX23-GFP Sequencing	X23GS1	5’-CTTGAGGAATCTAAAAATTGCTTCA-3’
	X23GS2	5’-GCAGATTTTGTAACCGTACAATTAGA-3’
	X23GS3	5’-TAGCCTCTTCCCTTCCGTTC-3’
	X23GS4	5’-CACAAATGATGTAGAAGAGAGGCA-3’
	X23GS5	5’-ACTAGAAGCGCGATCGTCATATGAGTAT-3’
	X23GS6	5’-TCTGCTCTGACTCTGTCCGA-3’
	X23GS7	5’-GGATGAGATTAGAGCTTCGC-3’
Lat52 promoter sequencing	Lat52 Pf	5’-**CGAGCTC** ACTCGACTCAGAAGGTATTGAGGA-3’
	Lat52 Pr	5’-**GGACTAGT** TTTAAATTGGAATTTTTTTTTTTGGTG-3’

based genotyping. After self-fertilization of *chx21*$^{+/-}$*chx23*$^{-/-}$ plants carrying the transgene, genotype of the T_2 generation was selected on hygromycin-containing plates and then tested by PCR using gene-specific primers of CHX21 and *chx21-2*. the resulting *chx21-2*$^{-/-}$ plants were further tested with gene-specific primers of CHX23, *chx23-4* and CHX23-GFP transgene (Table II-2). Finally all the *chx21-2*$^{-/-}$*chx23-4*$^{-/-}$ plants were confirmed to carry the CHX23-GFP transgene.

3-3. Molecular cloning and RNA expression

Genomic CHX23 cloning

The genomic region of the CHX23 gene, including the promoter region, the ORF without the stop codon, was amplified by PCR using primers CHX23GCf and CHX23GCr (Table II-2) using the BAC clone (F3F20_3) as template. attB1 and attB2 sequences for gateway recombination cloning were included in the forward and reverse primers, respectively. Gel-purified PCR product was cloned into pECHX23G after a BP reaction between the PCR product and the pDONR221 vector (Invitrogen, CA). Resulting clones were verified by sequencing. To link the genomic CHX23 with GFP for the protein localization, CHX23 genomic region was cloned into pMDC107-X23 after LR recombination reaction between pECHX23G and pMDC107 (Curtis and Grossniklaus, 2003). This plasmid has a Hyg resistant gene.

RNA isolation and RT-PCR.

Total RNA was isolated from seedlings or pollen of Aarabidopsis plants using Trizol reagent. Briefly, two week seedlings grown on 0.5 X Murashige and Skoog plates

and pollen from 6-week plants (Honys and Twell, 2003) were used. Isolated RNA were reverse transcripted using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Gene specific primers (Table II-2) were used to run a 30-cycle PCR. Actin 11 (At3g12110) was used as loading control and at the same time to distinguish any possible genomic DNA contamination.

3-4. Arabidopsis transformation

Agrobacterium tumefaciens strain GV3101 was transformed with the binary vectors pMDC107-X23 via electroporation and transformatants were selected on Luria-Bertani plates with gentamicin (50 µg/ml) and Kanamycin (50 µg/ml). Arabidopsis plants were transformed via Agrobacterium using the floral dip method (Clough and Bent, 1998). For the molecular complementation, chx21-2$^{+/-}$chx23-4$^{-/-}$ plants were transformed with a construct gCHX23-GFP. Sixteen T_1 seeds were sterilized and transformants were selected by hygromycin resistance on 0.5 X Murashige and Skoog plates containing hygromycin (30 µg/ml). Two-week-old hygromycin-resistant plants were genotyped by PCR methods using gene-specific primers. Three chx21$^{+/-}$chx23$^{-/-}$ 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 CHX23, *chx23-4* and CHX23-GFP transgene (Table II-2). Finally all the *chx21-2*$^{-/-}$*chx23-4*$^{-/-}$ plants were confirmed to carry the CHX23-GFP transgene. As the transgene in T1 is heterozygous, the expected genotype of hygromycin-resistant T2 population of CHX21$^{+/+}$, *chx21*$^{+/-}$ and *chx21*$^{-/-}$ would be 3:5:2.

3-5. Pollen assays

In vitro pollen germination

Pollen from freshly opened stage 13 flowers (Sanders et al., 1999) was collected and put in the liquid germination medium used by Fan et al (2001). Pollen was incubated in the medium at 25 ℃, 65% humidity and dark for six hours. Images were taken using Nikon microscope (Eclipse E600) under bright field light. Image J (free download from NIH) was used to analyze tube length.

Pollen DAPI/Alexander staining

Pollen from freshly opened stage 13 flowers (Sanders et al., 1999) was put on microscopic slides. Staining solution was immediately added to the pollen and images were taken using microscope (Nikon Eclipse E600) under UV (DAPI) or bright field light (Alexander).

Pollen fertility assay by limited pollination

Pollen from stage 13 flowers (Sanders et al., 1999) was collected and counted under a stereoscopic zoom microscope (Nikon SMZ1000). Then limited (e.g. 35) pollen grains were dabbed onto the stigma surface of a male sterile line, *imp1* (Park et al., 2002) (gift from Dr. Zhongchi Liu) flowers. Seven days after pollination, pods were examined under the microscope. Then 70% ethanol was used to de-color the pods and seeds inside were observed and counted. Finally pods were opened and seed images were taken directly under microscope.

Aniline blue staining of pollen tubes

Six hours after hand-pollination, the pistil was cut excised and fixed in 10% acetic acid overnight. Tissues were then softened in 1 M NaOH overnight, washed with 50 mM K-phosphate buffer (pH 7.5) and stained in 0.01% aniline blue. Images were taken using Nikon microscope (Eclipse E600) under UV light.

***in vivo* Pollen tube growth**

Hand pollination was performed as described above. After various times, pistils were dissected and the ovary wall was removed. Intact ovules were fixed in 80% acetone overnight, incubated overnight with X-Gluc for GUS activity, and examined by microscopy.

Semi-*in vitro* tube guidance

This method was modified from Palanivelu and Preuss (Palanivelu and Preuss, 2006). After hand pollination, pistil was cut at the shoulder region of the ovary. Cut pistils were incubated on the tube growth medium at 25 ºC for about 6 hours. Pollen tubes were incubated for 30 min in the GUS staining buffer at 37 ºC. Images were taken under bright field microscope (Nikon Eclipse E600).

GUS staining

Seedlings, flowers, pistils or pollen tubes were incubated in GUS staining buffer containing 50 mM sodium phosphate, pH 7.2, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.2% Triton X-100, and 2 mM 5-bromo-4-chloro-3-indolyl-b-D-GlcUA (X-Gluc) at 37 ºC for various periods (30 min to overnight). Images were taken

under the Nikon stereoscope (SMZ1000) or Nikon scope with differential interference contrast (Eclipse E600).

3-6. Microscopy

For the tobacco pollen, fluorescence images were taken under a Nikon E800 microscope (Tokyo, Japan) linked with a charge-coupled device camera (Spot, Diagnostic Instruments, Sterling Heights, MI). For both tobacco and Arabidopsis pollen, confocal images were taken with a Zeiss LSM 510 laser scanning confocal microscope. For stably-transformed Arabidosis plants, 10 independent T3 lines were checked and all showed the same GFP signal pattern.

4. RESULTS

4-1 CHX21 & 23 encode two homologous cation/proton exchangers

CHX21 & CHX23 are members of the AtCHX gene family, fourteen to eighteen of which are preferentially expressed in pollen (Sze et al., 2004), though their roles in pollen development and/or function were unknown. Bioinformatic analyses indicated *AtCHX21* (At2g31910) and *AtCHX23* (At1g05580) were products of a gene duplication: i) The two genes are located on chromosome I and chromosome II, respectively (Sze et al., 2004) in regions that had undergone segmental duplication; ii) Their coding sequences are interrupted by two introns located at identical (or conserved) sites of the coding region, although CHX23 has an extra intron; and iii) the deduced proteins are highly related to one another, as they share 82% (66%) similarity (identity) between the N-terminal transmembrane domains (Fig. II-1) and 80% similariy (identity) in the C-terminal hydrophilic regions.

4-2 Identification of *chx21 and chx23* T-DNA insertion mutant

To test the function of CHX21 and CHX23, single homozygous T-DNA insertion mutants of *chx21-2, chx21-s1* and *chx23-4* were obtained after verifying their genotype by PCR. To test if these are null mutants, total RNA was extracted and reverse transcribed from pollen and seedlings. Using three sets of primer pairs (Fig. II-2 A), CHX23 transcripts were expressed specifically in pollen from wild-type plants (Fig. II-2 B). However, *chx23-4* mutant contained a partial transcript corresponding to a sequence upstream of the T-DNA insertion. No transcripts were detected using primer pairs

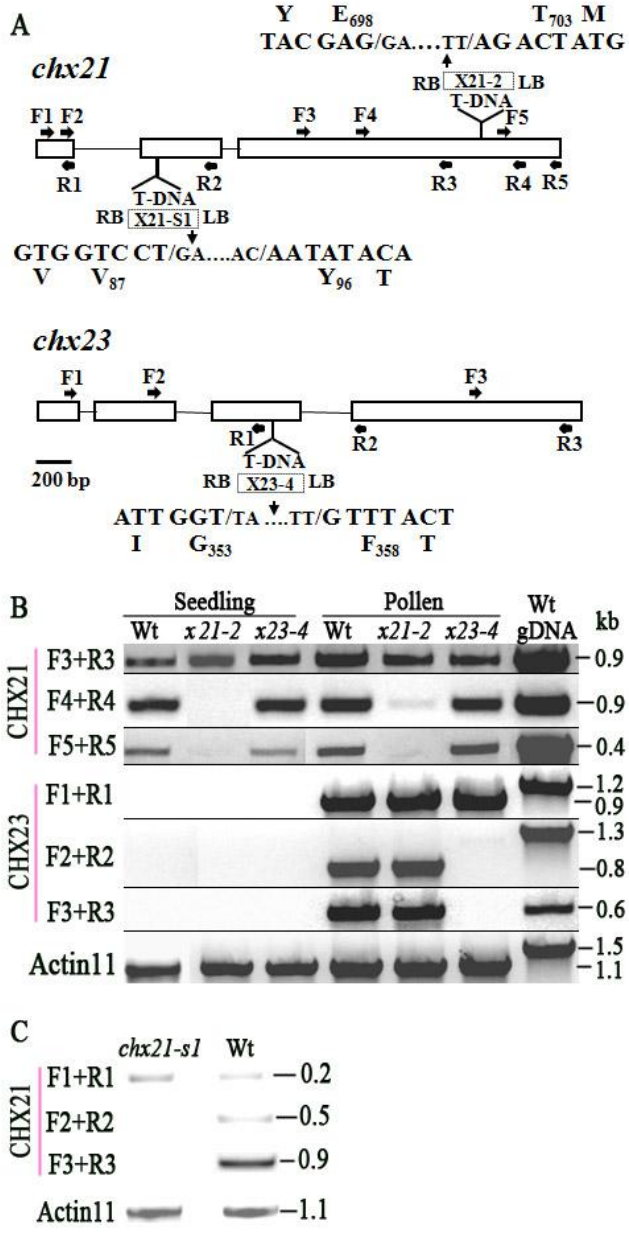
Fig. II-1. Protein sequence alignment of AtCHX21 and AtCHX23.

ClustalW2 was used for the alignment. Residues in red are the predicted transmembrane domains. CHX23 share 67% identical amino acids with CHX21. "*" means that the residues in that column are identical in both sequences in the alignment; ":" means conserved substitutions; "." means semi-conserved substitutions.

```
AtCHX21   MADPAAESIDAS-----SSRFGRVVCYNQTAMHG---------SNTISAAAPFFMTQLSV 46
AtCHX23   MSSGAPLNVTNPNYDIEESRFGKIVCYDQSLLFEKREQKGWESGSTLASSLPFFITQLFV 60
          *:. *. .:  .     .****::***:*: :.          ..*:::: ***:*** *

AtCHX21   ANLTYRILYYFLKPLCLPPFVAQILCGLLFSPTVLGNNEVVLKLIFPYKYTMLLETFANL 106
AtCHX23   ANLSYRVLYYLTRPLYLPPFVAQILCGLLFSPSVLGNTRFIIAHVFPYRFTMVLETFANL 120
          ***:**:***: :** ****************:****...::  :***::**:*******

AtCHX21   ALVYNVFLLGLGLDLRMIKIKDIKPVIIAIVGLLAALLAGAGLYYLPSNGEADKILAGCM 166
AtCHX23   ALVYNIFLLGLGMDLRMVRITELKPVIIAFTGLLVALPVGAFLYYLPGNGHPDKIISGCV 180
          *****:******:****::*.::******:.***.** .** *****.**..***::**:

AtCHX21   YWSIAFGCTNFPDLARILADLKLLRTDMGHTAMCAAVVTDLCTWILFIFGMAIFSKSGVR 226
AtCHX23   FWSVALACTNFPDLARILADLKLLRSDMGRTAMCAAIVTDLCTWVLLVFGFASFSKSGTW 240
          :**:*:.******************:***:******:*******:*::**:* *****.

AtCHX21   NEMLPYSLASTIAFVLLCYFVIQPGVAWIFNNTVEGGQVGDTHVWYTLAGVIICSLITEV 286
AtCHX23   NKMMPFVIITTAIFVLLCIFVIRPGIAWIFAKTVKAGHVGDTHVWFILGGVVLCGLITDA 300
          *:*:*: : :*  ***** ***:**:**** :**:.*:*******: *.**::*.***:.

AtCHX21   CGVHSITGAFLFGLSIPHDHIIRKMIEEKLHDFLSGMLMPLFYIICGLRADIGYMNRTVS 346
AtCHX23   CGVHSITGAFLFGLSIPHDHIIRNMIEEKLHDFLSGILMPLFYIICGLRADIGFMLQFTD 360
          **********************:************:****************:* : ..

AtCHX21   VGMMAVVTSASVMVKILSTMFCSIFLRIPLRDGLAIGALMNTKGTMALVILNAGRDTKAL 406
AtCHX23   KFMMVVVICSSFLVKIVTTVITSLFMHIPMRDAFAIGALMNTKGTLSLVVLNAGRDTKAL 420
          **.** .:*.:***::*:: *:*::**:**.:***********::**:**********

AtCHX21   DVIMYTHLTLAFLVMSMVVQPLLAIAYKPKKKLIFYKNRTIQKHKGESELCVLTCVHVLP 466
AtCHX23   DSPMYTHMTIALLVMSLVVEPLLAFAYKPKKKLAHYKHRTVQKIKGETELRVLACVHVLP 480
          *  ****:*:*:****:**:****:******** .**:**:** ***:** **:******

AtCHX21   NVSGITNLLQLSNPTKKSPLNVFAIHLVELTGRTTASLLIMNDEAKPKANFADRVRAESD 526
AtCHX23   NVSGITNLLQVSNATKQSPLSVFAIHLVELTGRTTASLLIMNDECKPKANFSDRVRAESD 540
          **********:**.**:***.**********************.******:********

AtCHX21   QIAEMFTALEVNNDGVMVQTITAVSPYATMDEDICLLAEDKQACFILLPYHKNMTSDGRL 586
AtCHX23   QIAETFEAMEVNNDAMTVQTITAVSPYATMHEDICVLAEDKRVCFIILPYHKHLTPDGRM 600
          **** * *:*****.: *************.****:*****:.***:*****::*.***:

AtCHX21   NEGNAVHAEINQNVMSHAPCSVGILVDRGMTTVRFESFMFQGETTKKEIAMLFLGGRDDR 646
AtCHX23   GEGNSSHAEINQNVLSHAPCSVGILVDRGMAMVRSES--FRGESMKREVAMLFVGGPDDR 658
          .***: ********:***************: ** **  *:**: *:*:****:** ***

AtCHX21   EALAYAWRMVGQEMVQLTVVRFVPSQEALVSAGEAADEYEKDKHVDEESIYEFNFKTMND 706
AtCHX23   EALSYAWRMVGQHVIKLTVVRFVPGREALISSGKVAAEYEREKQVDDECIYEFNFKTMND 718
          ***:********.:::********.:***:*:*:.* ***::*:**:*.***********

AtCHX21   PSVTYVEKVVKNGQETITAILELEDNNSYDLYIVGRGYQVETPVTSGLTDWNSTPDLGII 766
AtCHX23   SSVKYIEKVVNDGQDTIATIREMEDNNSYDLYVVGRGYNSDSPVTAGLNDWSSSPELGTI 778
          .**.*:****::**:**::* *:*********:*****: ::***:**.**.*:*:** *

AtCHX21   GDTLISSNFTMQASVLVVQQYSSANRQTAE----------------NNNQEPVQGKAKTD 810
AtCHX23   GDTLASSNFTMHASVLVIQQYSATKRQAAVTAAAATTVMGAVAGVTGNNLESAGGDAKMT 838
          **** ******:*****:****:::**:*                  .** *.. *.**

AtCHX21   HEA-TPFME------DEDDEVEHQYSMRR 832
AtCHX23   RDAHEPFMKSMYEDEDEDDEEDHQYGIHR 867
          ::*  ***:      ***** :***.::*
```

Figure II-2. Alleles of AtCHX21 and AtCHX23 T-DNA insertion mutants.

(A) Genomic structure of *chx21* and *chx23* showing T-DNA insertion sites. Boxes indicate exons. T-DNA insertion site and border sequences were verified by sequencing using both left and right border primers. (B-C) Absence of full-length transcripts in mutants: *chx21-2* and *chx23-4 (B) and chx21-s1 (C).* RNA extracted from seedlings (B) or pollen grains (B-C) was reverse transcribed and PCR-amplified (RT-PCR). gDNA refers to genomic DNA that was amplified as a positive control. Primers, F# and R#, correspond to sequences of CHX21 or CHX23 as shown in (A) and in Table II-2.


A
Y E698 T703 M
TAC GAG/GA....TT/AG ACT ATG
chx21
RB X21-2 LB
T-DNA
F1 F2 F3 F4 F5
R1 R2 R3 R4 R5
T-DNA
RB X21-S1 LB
GTG GTC CT/GA....AC/AA TAT ACA
V V87 Y96 T
chx23
F1 F2 F3
R1 R2 R3
T-DNA
200 bp
RB X23-4 LB
ATT GGT/TA ...TT/G TTT ACT
I G353 F358 T
B
Seedling
Pollen
Wt gDNA
kb
Wt x21-2 x23-4 Wt x21-2 x23-4
CHX21
F3+R3
F4+R4
F5+R5
CHX23
F1+R1
F2+R2
F3+R3
Actin11
0.9
0.9
0.4
1.2
0.9
1.3
0.8
0.6
1.5
1.1
C
chx21-s1 Wt
CHX21
F1+R1
F2+R2
F3+R3
Actin11
0.2
0.5
0.9
1.1

corresponding to regions spanning or downstream of the T-DNA insertion site (Fig. II-2 B). In wild-type plants, CHX21 transcripts were detected in RNA preparations of seedlings and in pollen. However, there were no full-length transcripts in seedling or from pollen of either *chx21-2* (SALK) or *chx21-s1* (SAIL) mutant (Fig. II-2 B & C). Partial transcripts corresponding to a region upstream of the T-DNA was also detected for *chx21-2* and *chx21-s1*. Thus these plants are likely null mutants of *chx21-2, chx21-s1* and *chx23-4*.

4-3 *chx21 or 23* single mutant pollen is fertile

Segregation of progeny from self-fertilized heterozygous single mutants was analyzed. T-DNA insertion in *chx21-s1* carries a BASTA resistance cassette. The F_1 progeny of *chx21-s1*$^{+/-}$ parent yielded BASTA^{R} seedlings at a predicted ratio of 3:1. The *chx23-4*$^{+/-}$ mutant produced F_1 with the following genotypes *chx23*$^{+/+:\ +/-:\ -/-}$ with a ratio of 32:43:28 near the expected ratio of 1:2:1 (Table II-3 i). These results indicated that pollen carrying a single mutation in either *chx21-s1* or *chx23-4* was functional and fertile. Furthermore, analyses of homozygous single mutants (*chx21-2* and *chx23-4*) showed that tube lengths of in vitro germinated pollen grains were similar to that of wild-type (Table II-4). Thus pollen carrying a mutation in either CHX21 or in CHX23 showed no defect in male fertility.

4-4 Double mutation of CHX21 and CHX23 blocked pollen-mediated gene transmission

To test if CHX21 and CHX23 proteins are functionally redundant, I tried to obtain double homozygous mutants. First, I identified plants *chx21-2* $^{+/-}$ *chx23-4* $^{-/-}$ that were

Table II-3. Disruption of both CHX21 and CHX23 genes causes defect in male-specific gene transmission.

Expt	Parent Female x Male	F_1 Segregation Genotypes		Expected	Observed ratio
i	*chx21-s1*$^{+/-}$ x self	Basta^{R}: Basta^{S}		3:1	341:111‡
	chx23-4$^{+/-}$ x self	chx 23 $^{+/+}$: $^{+/-}$: $^{-/-}$		1:2:1	34:43:28‡
ii	*chx21-2*$^{+/-}$ *chx23-4*$^{-/-}$ x self	chx21 $^{+/+}$: $^{+/-}$: $^{-/-}$	(chx23$^{-/-}$)	1:2:1	66:56:0*
	chx21-2$^{-/-}$ *chx23-4*$^{+/-}$ x self	chx23 $^{+/+}$: $^{+/-}$: $^{-/-}$	(chx21$^{-/-}$)	1:2:1	56:61:0*
iiia	*chx21-2*$^{-/-}$*chx23-4*$^{+/-}$ x WT	chx23 $^{+/+}$: $^{+/-}$	(chx21$^{+/-}$)	1:1	62:54‡
	WT$_i$ x *chx21-2*$^{-/-}$ *chx23-4*$^{+/-}$	chx23 $^{+/+}$: $^{+/-}$	(chx21$^{+/-}$)	1:1	119:1†
iiib	*chx21-2*$^{+/-}$ *chx23-4*$^{-/-}$ x WT	chx21 $^{+/+}$: $^{+/-}$	(chx23$^{+/-}$)	1:1	60:53‡
	WT$_i$ x *chx21-2*$^{+/-}$*chx23-4*$^{-/-}$	chx21 $^{+/+}$: $^{+/-}$	(chx23$^{+/-}$)	1:1	122:3†
iv	Complementation	T2 plant			
	chx21-2$^{+/-}$ *chx23-4*$^{-/-}$ with gCHX23-GFP$^{+/-}$ x self	chx21$^{+/+}$: $^{+/-}$: $^{-/-}$	chx23$^{-/-}$	3:5:2 Hyg^{R}	28:63:23‡

WT$_i$ refers to a male-sterile mutant (imp, At5g 42650) that is wild type for all other genes.

WT refers to wild-type (Col).

* Significantly different from the segregation ratio 1: 2: 1 (χ^2 , $p < 0.05$)

† Significantly different from the segregation ratio 1: 2: 1 (χ^2 , $p < 0.01$)

‡ Not significantly different from the Mendelian segregation ratio (χ^2 , $p < 0.05$)

Table II-4. *chx21*and *chx23* single mutant pollen showed similar tube growth with Wt Six hours after in vitro germination.

Pollen germination medium was the same as used by Fan et al (2001). Six hours after incubation at 25 ºC, pollen images were taken. Image J was used to analyze tube length. Results are from one experiment. Additional two independent experiments showed the same trend.

Pollen	Tube length (± SE, µm)	Sample #
chx21-2	372 ± 7	374
chx23-4	364 ± 9	210
Wt	350 ± 10	219

heterozygous for *chx21-2* and also homozygous for *chx23-4* and then examined their descendants after self-fertilization. Instead of the expected F1 population of $chx21^{+/+}chx23^{-/-}$, $chx21^{+/-}chx23^{-/-}$ and $chx21^{-/-}chx23^{-/-}$ at a ratio of 1:2:1, the observed results was close to 1:1:0, respectively (Table II-3-ii). Similarly, the progeny of self-fertilized $chx21\text{-}2^{-/-}chx23\text{-}4^{+/-}$ showed segregation distortion. No double homozygous mutants were recovered in either experiment. The segregation indicated a defect in one of the gametophytes.

To test whether the defect was due to male or female transmission, reciprocal crosses with the wild-type plant were made. When the pistil of the mutant $chx21\text{-}2^{-/-}$ $chx23\text{-}4^{+/-}$ was hand-pollinated with wild-type pollen, the progeny produced two genotypes of $chx21^{+/-}$ $chx23^{+/+}$ and $chx21^{+/-}$ $chx23^{+/-}$ with a ratio of 62:54 which is close to 1:1. However when pistils of wild-type (Wt_i) plants were pollinated with pollen from the $chx21\text{-}2^{-/-}chx23\text{-}4^{+/-}$ mutant, nearly all the progeny produced was the single heterozygous *chx21-2* mutant ($chx21\text{-}2^{+/-}chx23^{+/+}$). Mutant $chx21^{+/-}$ $chx23^{+/-}$ were rarely encountered (0.008%) (Table II-3-iii a). Similarly, when pistils of wild-type (Wt_i) plants were pollinated with pollen from the mutant $chx21^{+/-}$ $chx23\text{-}4^{-/-}$, 98% of the progeny was the single heterozygous *chx23-4* mutant ($chx21^{+/+}$ $chx23\text{-}4^{+/-}$) (Table II-3-iii b). Thus gene transmission through pollen is compromised. The genotype of the surviving progeny indicated that the haploid pollen carrying a single mutant of either *chx21-2* or *chx23-4* is functional while the double mutant (*chx21-2 chx23-4*) is not. These results also demonstrate that CHX21 and CHX23 proteins have overlapping function.

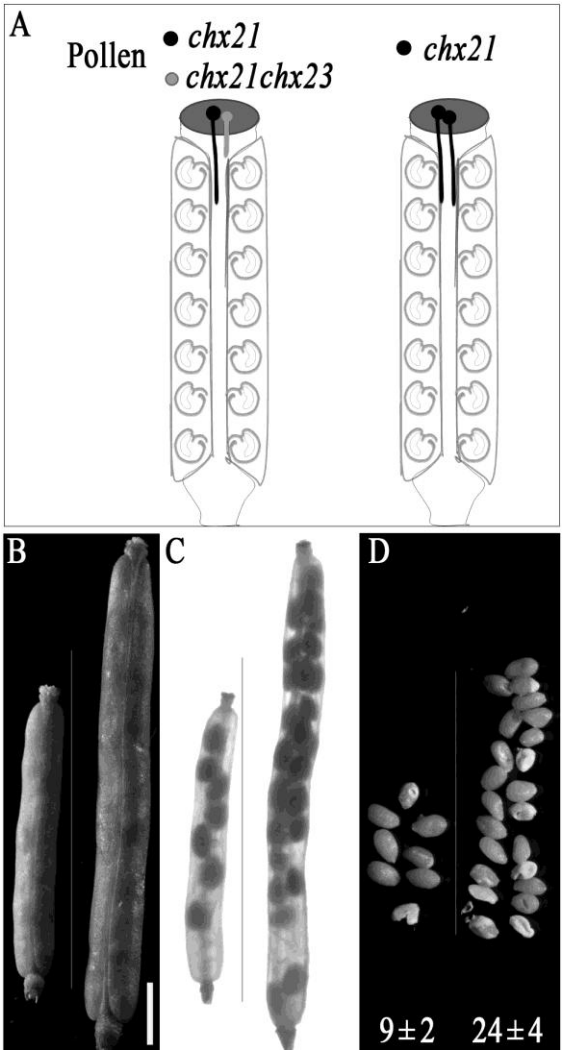
I tested if a wild-type CHX23 gene could restore fertility to double mutant pollen. Mutant *chx21-2*$^{+/-}$ *chx23-4*$^{-/-}$ plant was transformed with a construct that included the native CHX23 promoter, and the CHX23 ORF (without the stop codon) fused to a green fluorescence protein (GFP) gene at the C tail. T_1 plants were first selected by hygromycin-resistance and then PCR genotyped to identify the *chx21-2*$^{+/-}$ *chx23-4*$^{-/-}$ transformants that carry the transgene. After self-fertilization, the T_2 generation yielded plants with double mutant genotype *chx21-2*$^{-/-}$ *chx23-4*$^{-/-}$ at the expected ratio, indicating that the transgene CHX23 was able to complement the mutated *chx23-4* and restore function to double mutant pollen (Table II-3-iv). These results confirm that distorted gene segregation was indeed caused by mutations in CHX21 and CHX23. Furthermore, a wild-type CHX23 alone is competent in restoring pollen function.

***4-5 chx21chx23* double pollen is infertile**

It is possible that when pollen grains are in excess, 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. To test this idea, one pistil of a wild-type (Wt_i) flower containing about 50 ovules was given a limited number (e.g. 35) of pollen grains. If *chx21-2 chx23-4* pollen is slow but still competent, pollination with a mixture of *chx21-2* and *chx21-2 chx23-4* pollen (Fig. II-3, left) would produce a similar amount of seeds as pistils pollinated by *chx21-2* pollen alone (Fig. II-3, right). Seven days after pollination, the seed pods were visibly long and about 24 mature seeds were recovered from each pod in flowers pollinated with pollen from the single mutant *chx21-2 plants* (Fig. II-3). In contrast, seed pods were small and contained about 9 seeds per

Figure II-3. *chx21chx23* double mutant pollen is infertile.

(A) Diagram of limited pollination. WT pistil from the male-sterile *imp* line (Wt_i) was pollinated with 35 grains from either *chx21-2*$^{-/-}$ or *chx21-2*$^{-/-}$ *chx23-4*$^{+/-}$ parent. The *chx21-2*$^{-/-}$ *chx23-4*$^{+/-}$ parent should produce a 1:1 mixture of *chx21* single and *chx21chx23* double mutant pollen. (B) Seed pods from flowers given 35 pollen grains from *chx21-2* mutant (right) and from *chx21-2*$^{-/-}$ *chx23-4*$^{+/-}$ (left) at seven days after pollination (DAP). Scale, 1 mm. (C) Seed pods cleared of chlorophyll reveal less mature seeds in pistils given pollen from the *chx21-2*$^{-/-}$*chx23-4*$^{+/-}$ (left) than from *chx21-2* (right) mutant. (D) Number of seeds from each pod of a flower pollinated with 35 grains from *chx21-2* (right) or from *chx21-2*$^{-/-}$*chx23-4*$^{+/-}$ (left) plants. Representative results of three independent experiments.


A
Pollen
chx21
chx21chx23
chx21
B
C
D
9±2
24±4

pod when pistils were given 35 pollen grains containing a mixture of *chx21-2* and *chx21-2 chx23-4 pollen grains*. As ovules are in excess of pollen, the failure to produce more seeds using pollen from the chx21-2 -/- chx23 +/- plants suggested that chx21-2 chx23-4 pollen was not merely slow or less competitive than single mutant pollen, it was infertile.

4-6 *chx21chx23* double mutant pollen has normal germ-unit development

A loss in male fertility could be due to defective pollen development. Pollen grains collected from stage 13 flowers of *chx21-2*$^{+/-}$*23-4*$^{-/-}$ plants were examined by microscopy after DAPI staining. All the pollen grains consisted of three DAPI-stained nuclei and had similar sizes (Fig. II-4), so there was no indication for a defect in pollen development.

4-7 chx21chx23 double mutant pollen is viable

As DAPI is diffusible in both live and dead pollen cells, pollen grains collected from stage 13 flowers of *chx21-2*$^{+/-}$*23-4*$^{-/-}$ plants were examined by microscopy after Alexander staining for pollen viability. All the pollen grains showed purple color, which suggest that the double mutant pollen is viable (Fig. II-5).

4-8 chx21chx23 double pollen has normal in vivo germination and tube growth

To test if the male infertility is due to impaired pollen germination or tube growth, after limited pollination using 38 pollen grains, the length and number of pollen tubes within the transmitting tract were examined after aniline blue staining. The number of pollen tubes within the transmitting tract 6 h after pollination was 21 and 20 at a position T (about 600 μm below the stigma) with single or with a mixture of single and double

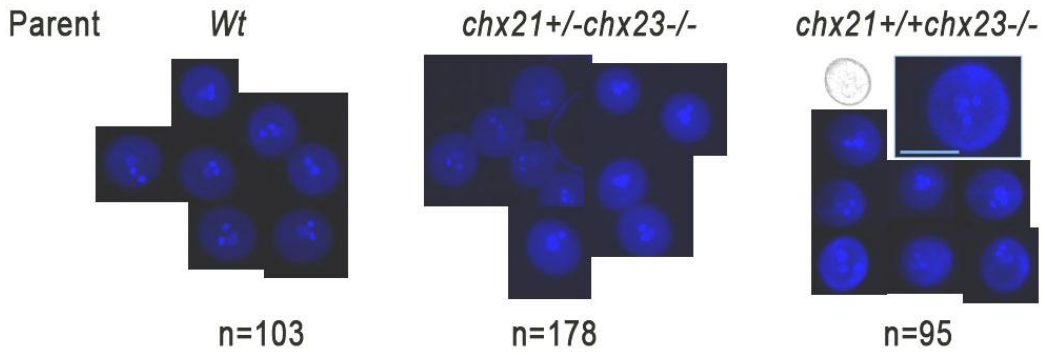


Figure II-4. Pollen grains from Wt, chx21$^{+/-}$chx23$^{-/-}$ and chx21$^{+/+}$chx23$^{-/-}$ were similar in their germ unit development.

Pollen grains from stage 13 flowers were collected by dapping flowers on a microscopy slide. DAPI solution was added and images were observed under UV. The three-nucleus germ-unit structure was found in all pollen grains from the three different plants. Scale, 10 µm.

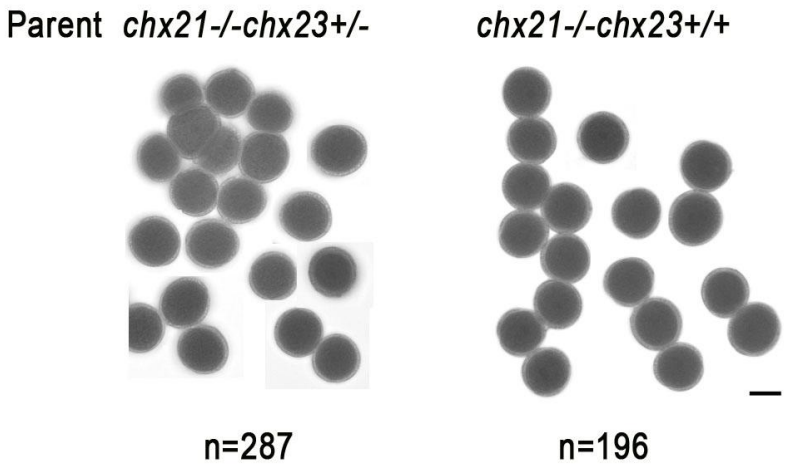


Figure II-5. Pollen grains from *chx21*$^{-/-}$*chx23*$^{+/-}$ stained were viable.

Pollen grains from stage 13 plants were collected by dapping flowers on a microscopy slide. Alexander staining solution was added before observation under bright field microscope. After staining, viable pollen will show purple cytoplasm, while dead pollen will be greenish. Scale, 10 µm.

mutant pollen used, respectively (Fig. II-6). Furthermore the lengths of the pollen tubes were quite similar based on the number of tubes (10) reaching the middle of the carpel 6 h after pollination with single or with a mixture of single and double mutant pollen. As half the pollen grains from the *chx21-2*$^{-/-}$*chx23-4*$^{+/-}$ plant should consist of double mutants which are infertile, the results suggested that double mutant pollen are competent in germination and in tube elongation in vivo similar to single mutant pollen.

4-9 Generating GUS-labeled *chx21chx23* double mutant pollen

To help visualize the double mutant pollen from single mutant, a mutant line *chx21-s1*$^{+/-}$ *chx23-4*$^{-/-}$ was generated (Fig. II-7). *chx21-s1* was obtained from a SAIL mutant collection (SAIL_162_A07) harboring T-DNA vector, pCSA110, which includes a BASTA resistance cassette, and the GUS gene under the control of the pollen-specific LAT52 promoter (Sessions et al., 2002). Thus the *chx21-s1* pollen expresses GUS activity and will turn blue (Fig. II-7-A), while *chx23-4* pollen remains colorless. The mutant was generated by crossing homozygous *chx21-s1* plant with *chx23-4*. The F_1 plant *chx21-s1*$^{+/-}$ *chx23-4*$^{+/-}$ was selfed and F_2 *chx21-s1*$^{+/-}$ *chx23-4*$^{-/-}$ plants were identified by BASTA resistance and by PCR-genotyping. This plant produces both GUS-carrying *chx21chx23* double mutant and non-GUS-carrying *chx23* single mutant pollen (Fig. II-7-B). To visualize double mutant pollen grown in vivo, after hand-pollination, pistils were cut and ovary wall materials removed. Intact ovules were stained for GUS activity (Fig. II-7-C).

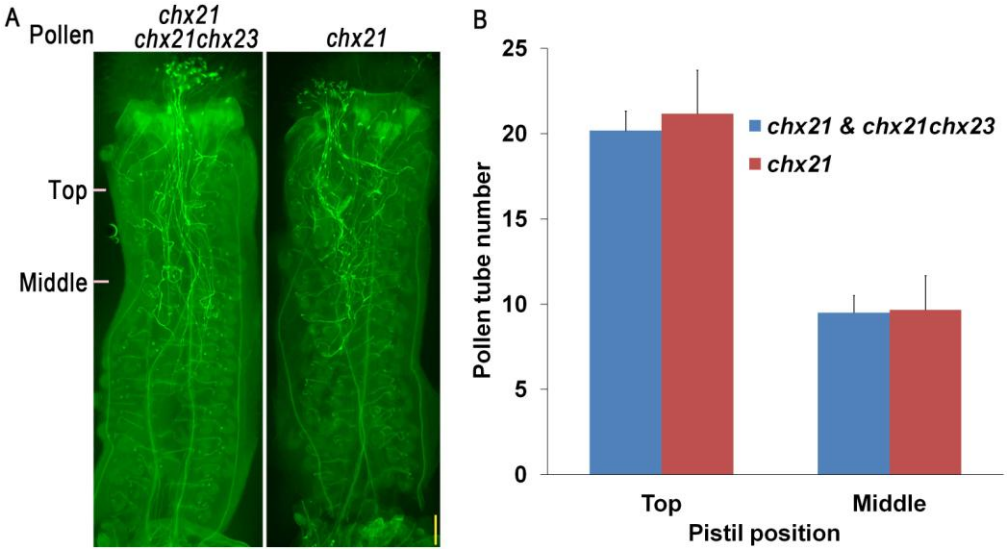


Figure II-6. In vivo tube growth is similar between *chx21* pollen and those with a mixture of *chx21* and *chx21chx23*.

Pollen from two parental lines, *chx21*$^{-/-}$*chx23*$^{+/+}$ and *chx21*$^{-/-}$*chx23*$^{+/-}$, were collected. Thirty-eight pollen grains from each were placed on the Wt_i pistil. Six hours later, the pistil was stained with aniline blue and the number of tubes scored at positions marked as 'Top' and 'Middle'. (A) Aniline blue stained tubes within the pistil. Scale, 200 μm. (B) Number of tubes visualized at positions 'Top' and 'Middle'. Results represent average of 6 independent experiments.

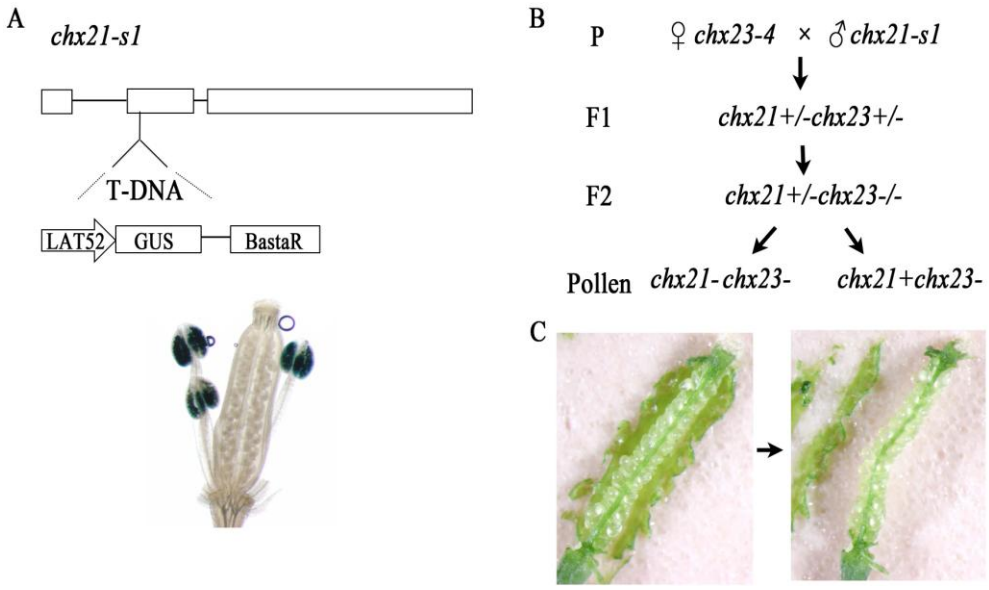


Figure II-7. Creating GUS-labeled *chx21chx23* double mutant pollen and isolating intact ovules for GUS staining.

The T-DNA inserted in *chx21-s1* carries a LAT52 promoter::GUS. *chx21-s1* mutant pollen turns blue after staining for GUS activity. (B) Strategy to distinguish double mutant (*chx21chx23*) from single mutant (*chx23*) pollen. *chx21-s1* mutant was crossed with *chx23-4* mutant, and the F1 was selfed. From the F2 progeny a plant with the genotype $chx21^{+/-}chx23^{-/-}$ was identified. Only double mutant (*chx21-s1chx23-4*), but not single mutant (*chx23-4*) pollen, expresses GUS activity and can be visualized after incubation with substrate. (C) Ovary showing partial (left) and complete (right) removal of the wall. After hand-pollination, the pistils were cut open to remove the ovary wall, and intact ovules were stained for GUS activity.

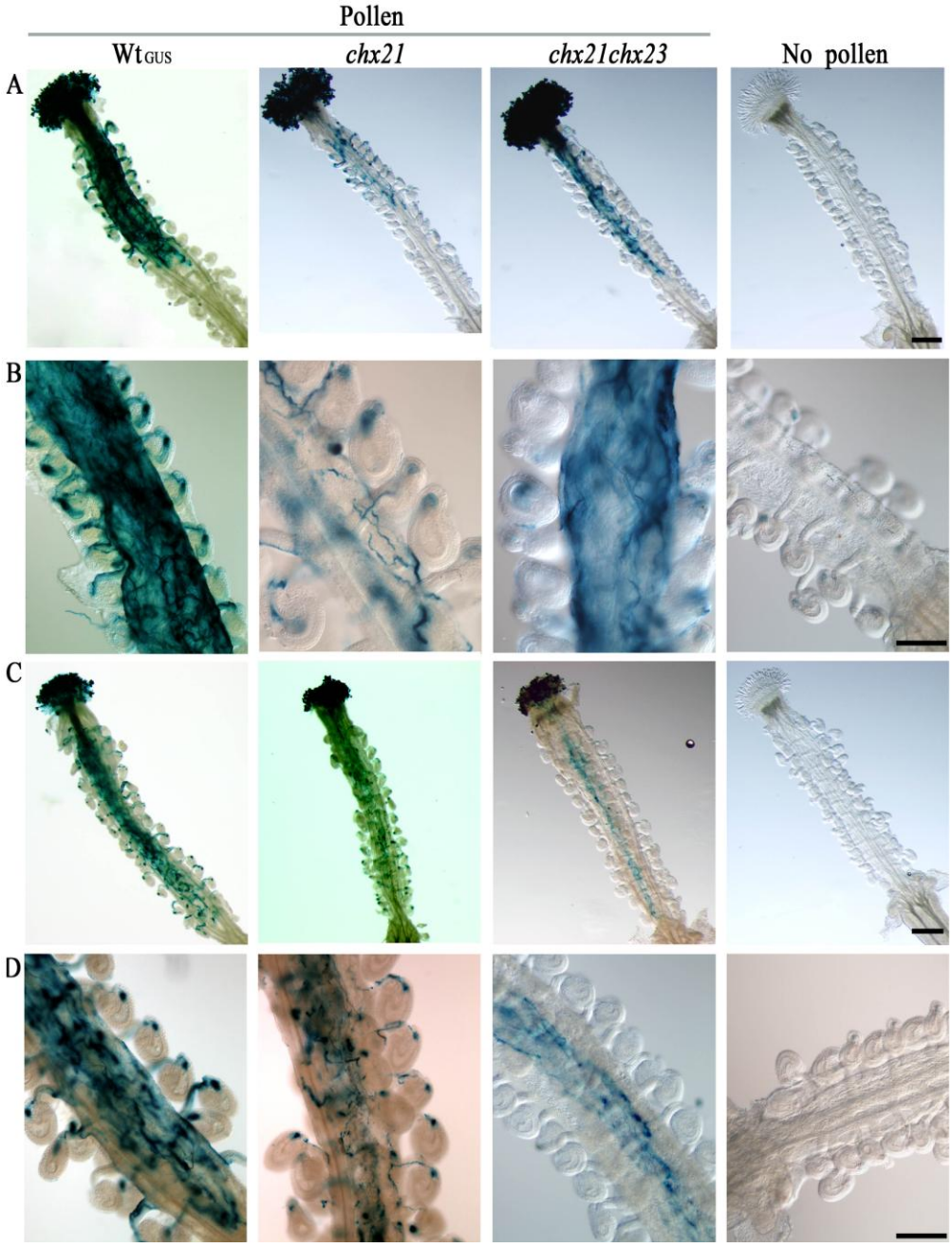
4-10 Double mutant pollen failed to turn towards the ovule in vivo

Pollen from *chx21-s1* and *chx21-s1*$^{+/-}$ *chx23-4*$^{-/-}$flowers were then tested for *in vivo* tube growth in pistils of wild-type plants (Wt_i). At 6 or 24 h after manual pollination, the ovary was excised to remove the carpel wall and then stained for GUS activity. At 6 HAP (Hours after pollination), wild-type (Wt_{GUS}) pollen grains from transgenic plants expressing Lat52 promoter::GUS had extended tubes more than half-way in the transmitting tract (Fig II-8-A). Furthermore, the ovules located in the top half of the ovary turned blue. Higher magnification indicated the presence of pollen GUS inside the ovules (Fig II-8B). Pollen from *chx21-s1* was similar (Fig. II-8A, 8B). By 24 HAP, most of the tubes had reached the bottom of the transmitting tract and all the ovules in the lower half of the carpel showed a GUS stain inside the ovule (Fig. II-8C, 8D). The turning of pollen tubes of single mutant at the funiculus and the presence of GUS inside all the visible ovules is striking, indicating the discharge of pollen content into the embryo sacs (Fig II-8D). Pistils that were not pollinated showed no GUS staining in the transmitting tract or the ovules.

After GUS staining, we observed that the *chx21-s1 chx23-4* double mutant pollen tubes were inside the transmitting tract, and tube lengths were equivalent or longer than that of wild-type tubes at 6 HAP (Fig. II-8A). This result would confirm the previous experiment (Fig. II-6) that double mutant pollen was able to germinate and to extend tubes in vivo. The tubes reached the bottom of the transmitting tract by 24 HAP (Fig. II-8C). Yet strikingly, none of the ovules showed GUS activity. The tubes stayed inside the transmitting tract and failed to reorient its growth to enter the funiculus (Fig. II-8B,

Figure II-8. ***chx21chx23*** **double mutant pollen failed to reach the ovule in vivo**.

In vivo pollen tube growth, tube reorientation and discharge into embryo sac. Wild-type pistils (Wt_i) were hand-pollinated with pollen of wild-type, single and double mutants. After 6 h (A-B) and 24 h (C-D), intact ovules were surgically removed and stained for GUS activity for 12 h. Frames B and D are higher magnification of A and C, respectively. Scale, 200 µm.


Pollen
Wt GUS
chx21
chx21chx23
No pollen
A
B
C
D

8D). Thus male infertility in *chx21-s1 chx23-4* double mutant pollen is due to a failure to shift the direction of pollen tip growth at the funiculus and to target the ovule, which suggests a failure in the guidance mechanism.

4-11 Recovering a rare chx21chx23 double homozygous plant

Even though the in vivo tube growth data showed that *chx21chx23* double mutant pollen fails to reach ovule (Fig. II-8), gene segregation data (Tab. II-3) showed that the double mutant pollen was competent, even though at very low frequency. A *chx21-s1*$^{-/-}$ *chx23-4*$^{-/-}$ double homozygous plant was recovered by chance (Fig. II-9), and this plant was sterile. The mutant plant showed normal vegetative growth and produced about the same number of flowers (Fig. II-9-i), but tiny or no obvious seed pod was found (Fig. II-9-ii). While Wt plant growing under same conditions produced about nine thousands seeds, only 9 seeds were recovered from the mutant plant (Fig. II-9-iii). Genotype of the double homozygous plant was confirmed by PCR-based genotyping using Gene- and T-DNA-specific primers (Fig. II-9-B).

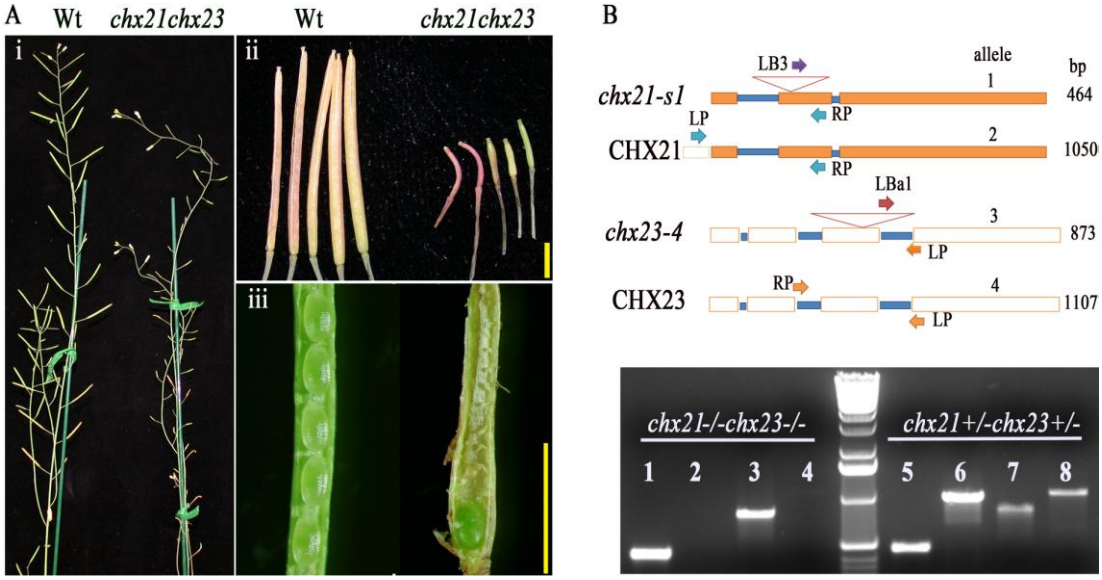
Furthermore, pollen (*chx21chx23*) from the new double homozygous plant showed normal germ unit development (Fig. II-10), competent in vivo germination and tube growth, but failed ovule targeting (Fig. II-11), which confirms the previous data. Pollen from this new double homozygous plant was used for the following pollen tube guidance assay.

4-12 Double mutant pollen fails to respond to guidance cues from isolated ovules

To test tube guidance in the *chx21chx23* double mutant pollen directly, a semi-in

Figure II-9. A rare *chx21-s1*$^{-/-}$*chx23-4*$^{-/-}$ double homozygous plant.

Seeds from *chx21-s1*$^{+/-}$*chx23-4*$^{-/-}$ parent were spread on soil. Then two-week seedlings were sprayed with Basta twice. The survival plants were screened for sterile phenotype, e.g. tiny seed pod, by eyes at six weeks after planting. A single plant out of several hundred was found to be sterile. Genomic DNA was isolated from this candidate plant and PCR was done to identify the genotype of the plant. (A) Phenotypes of the double homozygous mutant: i) The mutant at 6 weeks after planting; ii) Seed pods of mutant are extremely small (right); iii) Seed pod of Wt plant has full seed set (left), while out of 120 counted mutant pods, 191were empty, only 9 pod was found to contain a single full-sized seed in each (right) . Under the same growth conditions, a single wild-type plant is estimated to produce about 6 k seeds, but only 9 seeds were recovered from the double mutant. For the next four generations, these double homozygous seeds produced the same progenies as the parent: tiny pods and 5~10 seeds from each plant. (B) Double homozygous plant is a null mutant for both CHX23 and CHX21. Genotyping was done using Wt gene- and mutant allele-specific primers (arrows) with genomic DNA isolated from plant in (A) as template. The size of each PCR product and the ladder were indicated. Primers are shown in Table II-2.


A
Wt
chx21chx23
Wt
chx21chx23
i
ii
iii
B
LB3
allele
bp
chx21-s1
1
464
LP
RP
CHX21
2
1050
RP
LBa1
chx23-4
3
873
LP
RP
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2
3
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5
6
7
8

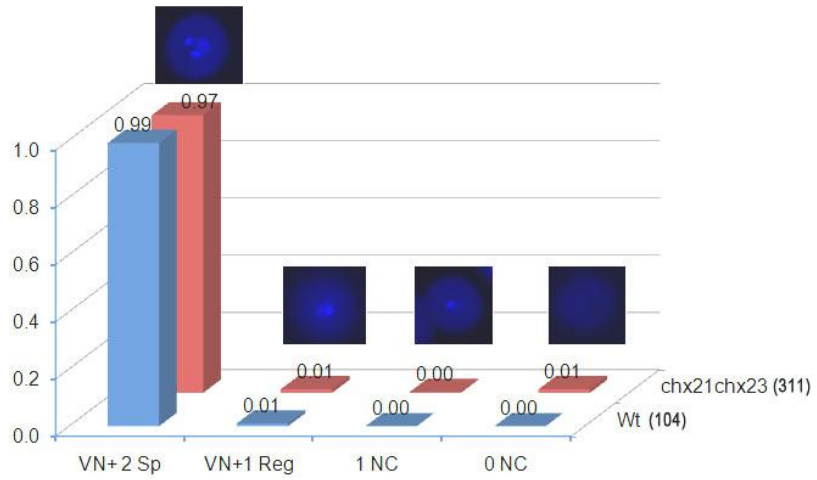


Figure II-10. ***chx21chx23*** **pollen from the rare** ***chx21-s1*** $^{-/-}$ ***chx23-4*** $^{-/-}$ **plant had normal Germ-unit development.**

Pollen grains from stage 13 flowers were collected by dapping a microscopy slide. DAPI staining was added before observation under UV light. VN, vegetative nucleus; NC, nucleus; Sp, sperm.

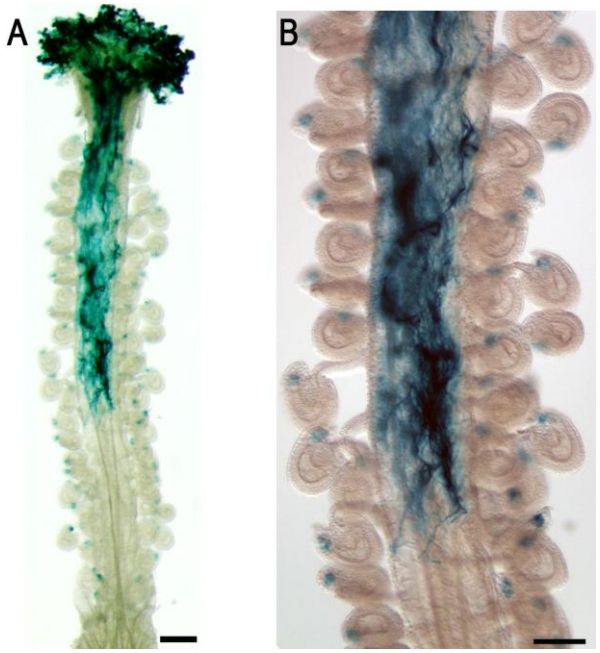


Figure II-11. Pollen from the *chx21-s1*$^{-/-}$*chx23-4*$^{-/-}$ plant failed to reach ovule.

The procedure was the same as described in Fig II-8. B is higher magnification of A. Scale, 100 μm.

vivo assay (Higashiyama et al., 1998; Palanivelu and Preuss, 2006) was conducted to see if isolated ovules could cause reorientation of the double mutant pollen tubes. Wild type pistils (Wt_i) were pollinated with Wt (Wt_{GUS}) or mutant pollen and then the ovary was severed at the shoulder and placed on tube growth medium for 6 h (Fig. II-12). Wild-type tubes emerging from the cut end of the ovary continue to grow on growth medium and show competent to orient their growth towards the ovule as shown before (Palanivelu and Preuss, 2006).

In 10 experiments, we found that 215 ovules out of 235 total ovules were targeted by a blue Wt_{GUS} pollen tube (Fig. II-13). However, when pollen from the *chx21-s1*$^{-/-}$ *chx23-4*$^{-/-}$ double homozygous mutant plant was used, only 21 out of 183 ovules were targeted by a blue *chx21chx23* pollen. In addition to observation by microscopy, Gus solution and buffer washes often caused untargeted ovules to float away while targeted ovules stayed tethered to the tubes. Thus there is a tight physical association of targeted ovule with the tube. Thus pollen tube response to guidance cues from the ovule depends on functional CHX21 and 23.

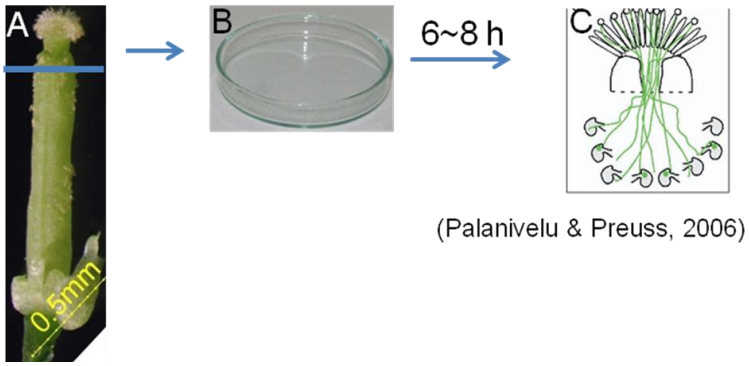


Figure II-12. Diagram of the semi in vivo tube guidance assay.

After hand-pollination, pistil was cut at the ovary shoulder position (A, line) and transferred to agar medium (B). Hours later, tube grew out of the cut pistil, continued grow and finally reached the surrounding ovule (C).

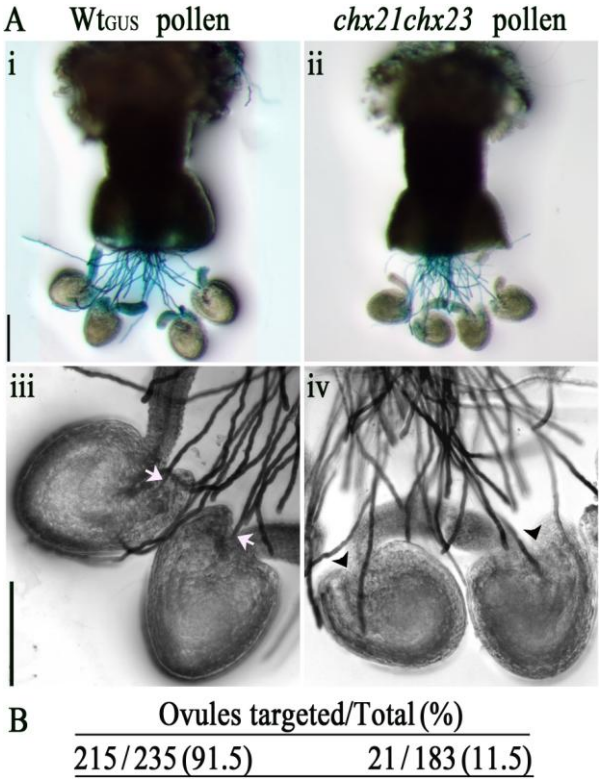


Fig. II-13. **Tube guidance in *chx21chx23* double mutant pollen was disrupted in a semi-in vivo assay.**

(A) Pollen from (i) wild type (Wt_{gus}) and (ii) *chx21chx23* double mutant were used to manually pollinate wild type (Wt_i) pistil, which was later excised and placed horizontally on plate. Pollen tubes emerging from the cut end were visualized by GUS staining. (iii-iv) Magnified view of ovules from experiment in (i-ii). Arrows mark two tubes penetrating ovules through the micropylar region; Arrowheads mark the micropylar region on untargeted ovules. Scale, 100 µm. (B) Table showing ratio of targeted ovules using Wt_{gus} pollen (left) and *chx21chx23* pollen.

5. DISCUSSION

The CHXs are predicted cation/proton exchangers based on sequence similarities to known transporters in yeast and bacteria (Sze et al., 2004). Even though most of the CHXs are preferentially or specifically expressed in pollen, their role in plant reproduction is completely unknown. Here, I show genetic evidence that CHX21 and CHX23 play a key role in pollen tube guidance. Pollen that is defective in both genes is infertile due to failed ovule targeting.

5-1 Primary findings of this study

CHX21 and CHX23 play redundant but essential roles in male fertility. Single *chx21* or *chx23* mutant pollen was fertile like wild-type as shown by the normal segregation in the descendants of heterozygous plants and the normal tube growth in vitro, however the double *chx21chx23* mutant was infertile based on segregation ratio analyses and hand-pollination with limited number of pollen grains. Infertility was not caused by any defect in development of the pollen grains as shown by the morphology and size of the grains, the viability as shown by Alexander staining, and the presence of three DAPI-stained nuclei.

My findings give clues regarding which phase of pollen tube growth and guidance has been impaired in the *chx21chx23* double mutant. Pollen tube growth and guidance has been divided into six phases: i) stigma penetration, ii) growth in the transmitting tract, iii) transmitting tissue exit, iv) funicular guidance, v) micropylar guidance and ovule targeting, and vi) sperm release (Johnson & Lord 2006). Using mutant carrying a T-DNA insertion with a pollen-specific promoter driving the GUS reporter, I showed that

chx21chx23 double mutant pollen grains are able to germinate and extend a tube in the transmitting tract, suggesting there is no apparent defect in the early stages of tube growth and guidance (Fig. II-8). As double mutant tubes grow to the bottom of the transmitting tract and fail to turn at the funiculus, my results using intact pistils do not distinguish whether the defect is due to inability to exit the transmitting tract or inability to turn at the funiculus. Using the semi-in vivo assay, I showed that double mutant pollen emerging from excised style still failed to target isolated ovules. As the barrier to exit the transmitting tract is removed in excised styles, these results indicate that failure of the double mutant pollen to target ovules is probably due to inability to sense and/or respond specifically to funicular and micropylar guidance cues.

In very rare cases, a double mutant pollen was fertile and produced a fully developed seed that produced a viable yet double homozygous mutant plant. The ability to produce a fully-developed seed by pollen that rarely reaches ovule would indicate that the last step, the sperm release, and union with the egg were not impaired in the double mutant pollen. Reciprocal crosses had shown that the functions of the female gametophyte did not depend on *CHX21* and *CHX23* genes.

At this time, I cannot eliminate the possibility that double *chx21chx23* mutant failed to get primed completely as the 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 to perceive and respond to ovule cues (Higashiyama et al. 1998; Palanivelu & Preuss 2006). Regardless of whether the sporophytic, gametophytic or both phases are affected by

CHX21 or CHX23, my results point to a critical role of these gene products in sensing and/or responding to guidance cues to enable precise targeting of pollen tubes to ovules.

5-2 Contribution to the field

To deliver sperm cells to the ovule for fertilization, pollen tube needs guiding cues from the female cells along the growth pathway. Female cells that play guiding roles are being identified (Johnson and Lord, 2006), and candidate guiding molecules from these female cells are being proposed (Higashiyama and Hamamura, 2008; Okuda et al., 2009). However, how the pollen tube senses and responds to the guiding signals is poorly understood. Mutants whose pollen tubes fail to find the ovule had been reported before, however most are defective in either the development or functions of the female gametophyte (Hulskamp et al 95; Ray et al 97; Shimizu et al 2000). To my knowledge, only a small number of male gametophyte mutants have shown failure in pollen tube function (Palanivelu et al, 2003; Von-Besser et al, 2006).

Gametophytic mutants are difficult to analyze, as they must be maintained as heterozygotes plus it is challenging to distinguish mutant pollen from wild-type ones. A forward genetic screen of mutants expressing the pollen-specific GUS reporter yielded several single hap mutants altered in male gametophyte development or tube growth (Johnson et al 2004). Three mutant pollen tubes failed to exit the transmitting tract. The wild-type gene of one, HAP1 (At1g02140), encodes mago nashi, an ortholog of a Drosophila gene required to establish oocyte polarity. Another mutant pollen, *hap2*, showed reduced targeting of the ovule, though Hap2 (GCS2) encodes a conserved, sperm-specific membrane protein that is required for fertilization as well as pollen tube

guidance (von Besser et al. 2006). I took a reverse genetics approach, and have discovered the first double mutant where a primary defect is failed perception, response or both to guidance cues from the ovule. I also took advantage of T-DNA insertion mutants expressing the pollen-specific GUS reporter to distinguish the double mutant pollen and reveal the defect in pollen tube guidance.

Interestingly, the defect of the double mutant pollen is caused by null mutations in two paralogous CHX genes that encode cation/H^+ cotransporters. The CHX gene family is found in all flowering plants sequenced so far (Sze et al 2004; Genevestigator), suggesting their roles are critical for plant survival. My results support the idea that gene duplications in plants result in multiple genes with overlapping functions and ensure completion of essential processes, like reproduction. This might explain why forward genetic screens have yielded only a small number of male gametophyte mutants. To my knowledge, this is the first report to demonstrate a signaling role of two related transporters in the perception and/or transduction of ovule cues that control guidance of pollen tube growth *in vivo*. This unexpected finding is significant in two ways: i) it will facilitate the identification and integration of other signaling components critical for pollen perception and response to female cues, and ii) importantly, it will shed new light on the molecular mechanism of redirected polarized tip growth not only in plant, but also in other eukaryote cells that exhibit polarity.

5-3 Role of CHX23: Discrepancies with other published work

The CHX23 function as revealed by this study differs in several significant ways from a previous report: i) Song et al (2004) showed that CHX23 is vegetative cell-

specific based on promoter-driven GUS activity and RT-PCR; while my results and those from the Sze lab showed that CHX23 is a expressed specifically in pollen based on promoter-driven GUS and RT-PCR (Sze et al, 2004); ii) Song et al (2004) showed that fluorescence signals from GFP-tagged CHX23 driven by the strong Ca35S promoter was localized to chloroplasts, while my results showed the protein localized to ER in pollen tube either driven by the pollen-specific Lat52 promoter or the CHX23 native promoter, or to prevacuolar compartment (PVC) when transiently expressed in protoplasts; iii) Using RNA-mediated interference, they suggested CHX23 played an important role in chloroplast development and function. I used single and double mutants to show that CHX23 had an overlapping role with CHX21 in pollen tube guidance. Only double mutant pollen failed to target ovule.

After careful examination of the paper, major concerns about the study from Song et al (2004) are as follows:

1) The promoter region used for CHX23 expression studies is suspect **,** as they did not provide primer information used for the CHX23 promoter cloning. The identity of the RT-PCR product is uncertain, as the size was not verified by mol size ladder and related CHXs share considerable sequence homology.

2) Protein subcellular localization. Even though the primers they used for the cDNA cloning is correct. No information is available to show if seqeuencing was done to check the clone. Chloroplast imaging is especially difficult in that this organelle shows strong autofluorescnece under confocal, which could be easily misinterpreted.

3) The specificity of the sequence used for dsRNA-mediated interference is suspect. For instance, the reverse pimer they used for making dsRNA construct is not specific for CHX23, and overlaps extensively with CHX21. So the dsRNAi could interfere with other CHXs. Furthermoe, none of the T-DNA insertion mutants, *chx23-4/ chx23-4* single or *chx21-2/chx21-2, chx23-4/chx23-4* double mutants showed the ”yellowish” and ”small size” phenotypes shown by Song et al (2004). Vegetative growth of the single or double mutants were similar to wild type plants .

Overall, results from Song et al (2004) is not consistent with results from my study.

III. DETERMINE THE MEMBRANE LOCALIZATION AND BIOCHEMICAL FUNCTION OF CHX23 IN POLLEN

1. ABSTRACT

To understand the cellular roles of CHX21 and CHX23 in pollen tube guidance, the subcellular localization and transport activity of CHX23 were determined. By green fluorescence protein (GFP)-tagging, CHX23 transiently expressed using a strong pollen-specific promoter was localized on the ER in growing tobacco pollen tubes. Under its native promoter, CHX23-GFP showed similar reticular membrane localization in stably transformed Arabidopsis pollen tubes, confirming that CHX23 is associated with ER. To test the transport activity, CHX23 was heterologously expressed in bacterial cells defective in K^+ uptake. Expression of CHX23 enhanced cell growth under low K^+ and alkaline pH conditions. CHX23-transformed cells accumulated more K^+ than vector only-transformed cells, and this effect is pH-sensitive, more prominent at alkaline pH. This suggests that CHX23 has a role in regulating K^+ and H^+ homeostasis. Combined with the genetic results, the ER-localized CHX21 and CHX23 are proposed to facilitate shifting the polar growth axis to re-orient pollen tube growth and target an ovule by changing the local cellular pH.

2. INTRODUCTION

In flowering plant reproduction, the male gametophyte must overcome physical barriers to reach the highly protected ovule. After landing on a compatible stigma, each pollen tube grows rapidly by tip growth to deliver the sperm cells to the ovule with high precision. The cellular and molecular bases of polarized tip growth in vitro have been studied for several decades. To sustain fast cell growth, large amount of new membrane and wall materials need to be added to the growing tip. Pollen tube has highly active vesicle trafficking and organelle dynamics. Even though the number of molecular players involved has steadily increased, the establishment and maintenance of polarized tip growth is still poorly understood.

Studies using both electron and fluorescence microscopy showed a high density of small vesicles accumulate at the tube apical region (Steer and Steer, 1989; de Graaf et al., 2005). After exocytosis, these vesicles are thought to release new wall materials and provide new membranes to the growing site. Active endocytosis at the tip region was also suggested, as the amount of materials released by exocytosis exceeded the amount needed for growth (reviewed in Steer and Steer, 1989). Indeed, indirect (Derksen et al., 1995; Blackbourn and Jackson, 1996; Lind et al., 1996) and direct (Parton et al., 2001) evidence showed highly active endocytosis in the tip region of growing pollen tube. For example, one Rab GTPase, a key regulator for vesicle trafficking, was found to be essential for the membrane trafficking at this region and for the tip-focused tube growth. In tobacco pollen, green fluorescence protein (GFP)-labeled NtRab11 is predominantly localized to the pollen tip. Altering the activity of NtRab11 inhibited both exocytosis and

endocytosis pathways. As a results, both polar cell growth and total tube growth rate were reduced and male fertility was impaired (de Graaf et al., 2005).

In addition to active vesicle trafficking, the ER shows dynamic movements in pollen tubes grown *in vitro*. The ER of lily pollen was monitored by GFP-labeled with the ER retention sequence HDEL or using cytochalasin D tetromethylrodamine (Lovy-Wheeler et al., 2007). ER moved in an oscillatory manner into and out of the tube apex along the actin filament. Cross-correlation analysis showed that oscillatory ER movement led oscillatory tube growth, suggesting dynamic ER in the apical region is related to the tip growth, but the mechanism is unknown. Other organelles, like vacuole (Hicks et al., 2004; Lovy-Wheeler et al., 2007) and mitochondria (Lovy-Wheeler et al., 2007), also show highly dynamic movements in growth pollen tube. Mitochondria showed an ER-similar oscillatory pattern below the subapical region. In contrast, the tubular-looking vacuoles moved in the shank region and far back from the apical clear zone, no oscillatory pattern was found in the vacuolar movements (Lovy-Wheeler et al., 2007).

The dynamic cytoskeleton is critical for the directed movement of small vesicles and organelles in pollen tube. Both small vesicles at the apical region and the organelles in the shank region move along actin filaments (Cheung and Wu, 2008). Actin filaments arrange in different forms in tube regions. For example, actin in the subapical region showed a ring-like structure (Lovy-Wheeler et al., 2005), while in the apical region, actin forms thin and short bundles (Fu et al, 2001). Interestingly, dynamics of the tip actin filaments oscillate and lead tip growth (Fu et al, 2001). Applying actin depolymerizing

drugs inhibited tube growth (Vidali and Hepler, 2001), which suggests the crucial role of actin in pollen tip growth.

Actin binding proteins (ABPs) bind to and affect the arrangement and activity of actin. Three types of ABPs have been found in pollen (Yokota and Shimmen, 2006), among which, the activities of profilin and Gelsolin family proteins are regulated by Ca^{2+}. Higher Ca^{2+} enhances binding of profilin and Gelsolin family proteins to actin and their depolymerizing activities. For the third type of ABPs, the ADF/cofilin, the activity is pH sensitive. Alkaline pH enhances activity of ADF to sever actin filaments. Pollen tube subapical region forms an alkaline band, where the cable actin discontinued from the shank region and instead, actin forms ring structure. Higher ADF activity could be the cause of this significant actin arrangement change. Thus, transporters regulating cellular Ca and pH and might play important roles in regulating vesicle dynamics and organelle movement, though the molecular bases of ion dynamics in pollen is largely unknown.

Studies to understand the roles of transporters in pollen development and function are just beginning (Sze et al 2006). Several transporters have been functionally identified in pollen as important player in pollen development (Dettmer et al., 2005), germination (Sivitz et al., 2008), or tube growth (Mouline et al., 2002; Schiott et al., 2004; Frietsch et al., 2007). Curiously, transporter genes, such as the CHX family, are preferentially or selectively expressed in pollen though their function and multiplicity are not understood (Sze et al 2004; Bock et al 2006).

In chapter II, I used a reverse genetic approach to demonstrate that double chx21chx23 mutant pollen was competent in pollen germination and tube growth but

infertile. Infertility was due to failure of tubes to target ovule, apparently due to inability to sense and/or respond to guidance cues and reorient the direction of tip growth.

To understand the subcellular bases of CHX action, I determined the membrane location of CHX23 in pollen and its activity after expression in E coli. Here I showed that CHX23 is localized to an endomembrane resembling the reticulate ER of growing pollen tubes. CHX23 activity was tested after expression in bacteria that lack K^+ uptake systems. CHX23 expression enhanced cell growth under low K^+ and alkaline conditions and increased K^+ content. These results suggest a potential role of CHX23 in altering cellular pH and K^+ homeostasis. Combined with the biological function of CHX23 in pollen tube guidance, this research suggests a key role of the regulation of cellular micro-domain pH and/or osmosis in polar cell growth and reorientation.

3. MATERIALS AND METHODS

3.1 Yeast and bacterial cells

Yeast mutants KTA40-2 and LMM04 were used to express CHX23. Compared to KTA40-2, mutant AXT3 and LMB01 contain one more known cation/proton exchangers, KHA1 and NHX1, respectively. AXT3 and LMB01 were used as control in the cell growth assay, to compare any possible functional similarity between CHX23, KHA1 and NHX1.

E. coli strain LB2003, which lacks the three K+ uptake systems (Stumpe and Bakker, 1997), was used to assay activity of CHX23. Genotypes of these yeast and bacterial cells were shown in Table III-1.

3-2. Molecular cloning

CHX23 cDNA in Gateway entry vector

Total RNA was isolated from Arabidopsis mature pollen and the first-strand cDNA was synthesized using reverse transcriptase. Primers CHX23Cf and CHX23Cr (Table. II-2) were used to amplify the cDNA. The forward and reverse primers contain the attB1 and attB2 sequences for gateway recombination cloning. Gel-purified PCR product was cloned into the pECHX23 after BP reaction with the pDONR221 entry vector (Invitrogen, CA). Resulting clones were verified by sequencing. Compared to the predicted full length cDNA (2604 bp), the cloned cDNA is short 24 bases at the 3' end. Thus the deduced protein is near full-length with a truncation of 7 amino acids at the C terminus. (The cDNA was used for protein localization in plant cells and for functional assay in yeast.)

Table III-1. Yeast and bacterial strains used in this study.

Strain Name	Genotype	References
	A**. Yeast cells**	
KTA40-2	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mall0 ena1*D*::HIS3::ena4*D *nha1*D*::LEU2 nhx1*D*::TRP1 kha1*D*::kanMX*	Maresova and Sychrova, 2005
LMM04	*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mall0 ena1*D*::HIS3::ena4*D *nha1*D*::LEU2 trk1*D*::LEU2 tra2*D*::HIS3 kha1*D*::kanMX:tok1*D *NHX1*	Maresova and Sychrova, 2005
AXT3	Δ*ena1-4::HIS3* Δ*nha1::LEU2* Δ*nhx1::TRP1* *KHA1*	Quintero et al, 2000
LMB01	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mall0 ena1*D*::HIS3::ena4*D *nha1*D*::LEU2 kha1*D*::kanMX* *NHX1, NHA1*	Maresova and Sychrova, 2005
	B. **Bacteria cell**	
E. coli LB2003	(F^- *kup1* Δ*kdpABC5* Δ*trkA rpsL metE thi rha gal*	(Stumpe and Bakker, 1997)

CHX23-447aa-ΔC cDNA (used in yeast)

A carboxyl tail-truncated CHX23 cDNA was constructed using primers CHX23CΔf and CHX23CΔr with pECHX23 as template (see Table II-2 for primer sequences). The forward and reverse primers contain the attB1 and attB2 sequences for gateway recombination cloning. Gel-purified PCR product was cloned into the pECHX23ΔC after BP reaction with the pDONR221 vector (Invitrogen, CA). Resulting clones were verified by sequencing. The deduced protein has 447 amino acids and lacks the C hydrophilic tail of 420 Amino acids. (This clone was used for expression studies in yeast cells).

Yeast expression vector

Entry clones pECHX23 and pECHX23ΔC were used to have LR reaction with the yeast vector pYESDR196, and thus produced yeast expression clones pYESDR196-CHX23 and pYESDR196-CHX23ΔC, respectively.

Bacterial vector pPAB404-CHX23

CHX23 cDNA was amplified using primers AtCHX23-BglII-SE and AtCHX23-XhoI-AN (Table II-2), with pECHX23 as template. PCR product was gel-purified and inserted into vector pPAB404 after ligation. The ligation mixture was used to transform E. coli XL10 Gold, and transformants were plated on LB-ampicillin medium at 37 ºC overnight. Colony PCR and sequencing were performed. A sequence that is truncated after aa I778 was obtained. [Construct by Lalu in Uozumi lab]

Plant vectors

CaMV 35S promoter driven CHX23-GFP/RFP vectors

To localize protein in plant protoplasts, CHX23 was recombined into the gateway vectors that have CaMV 35S promoter driven GFP/RFP. Entry clone pECHX23 was used in the LR reaction with the Gateway destination vectors, p2GWF and p2GWR, to produced the expression clones p2GWF-CHX23 and p2GWR-CHX23, respectively.

LAT52-driven GFP/RFP vector (for protein localization in pollen)

To localize protein in pollen, CHX23 was placed into a modified gateway vector with a pollen-specific promoter. Primers Lat52 Pf and Lat52 Pr (Table. II-2) 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 Sac I and Spe I sites, respectively. Gel-purified PCR product and vectors p2GWF7 and p2GWR7 were digested with Sac I and Spe I, gel-purified, and ligated, resulting in new vectors pLatGWF7 and pLatGWR7, respectively. The inserted Lat52 promoter sequence in the new vectors was confirmed by sequencing. After LR recombination reaction between pECHX23 and the newly constructed Lat52 vectors, CHX23 cDNA was placed into the new vectors giving pLatGWF7-X23 and pLatGWR7-X23, respectively.

3-3. Cell transformation and growth assay

Arabidopsis leaf protoplast

Isolation and transformation of Arabidopsis mesophyll protoplast was based on Yoo et al., (2007). Leaves from 3-week-old wild-type *Arabidopsis thaliana* (ecotype

Col-0) grown under 12 h light at 50-150 µE were used to isolate the protoplast after cell wall enzyme digestion. Freshly isolated protoplasts were transformed with DNA construct in a PEG/Ca solution. For each transformation, about 10 µg plasmid of about 5 kb in size and 150 µl protoplast with a cell density of 2.5×10^5/ml was used. After incubate at room temperature for overnight, cells were observed by confocal microscopy and images were taken.

Tobacco pollen bombardment

Pollen bombardment method was based on Chen et al (2002). pLatGWF-X23 and pLatGWR-X23 plasmids were prepared using the QIAGEN plasmid midi kit (Qiagen, Valencia, CA). For each bombardment, 1.25 µg DNA and 7 mg tobacco (Nicotiana tabacum) pollen was used. After bombardment, pollen grains were cultured on medium contains 1mM KNO_3, 1.6 mM H_3BO_3, 0.8 mM $MgSO_4$, 3 mM $Ca(NO_3)_2 \cdot 4H_2O$, 8% sucrose, 0.7% agarose, and 10 mM MES pH 6.0. After 4 h incubation, pollen tubes were observed under fluorescent (Nikon E800) and confocal (Zeiss LSM 510) microscopes and images were taken.

Arabidopsis stable transformation with CHX23::CHX23-GFP

The method was described in chapter II, section 3-4.

Yeast transformation and growth assay

Thawed competent cells were transformed with the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). Transformant cells were grown on YNB + MA plates at 30 ºC for 3 ~ 4 days.

For the growth assay, two to three colonies were picked and incubated in YNB + MA liquid medium supplied with glucose at 30 ºC for overnight. Cells were then diluted 7 times into YNB + MA liquid medium depleted glucose and grow at 30 ºC for overnight. Cells with the same O.D value were then spotted on YNB + MA plates with different pH, K^+, Na^+ and hygromycin conditions. Images were taken 2 days after cell growth.

Bacteria transformation and growth assay

pPAB404-CHX23 plasmid was used to transform E. coli strain LB2003, which lacks the three K^+ uptake systems (Stumpe and Bakker, 1997). Growth tests of the transformed strains were carried out as described by Matsuda et al (2004). Transformant cells were grown at 30°C on synthetic solid K30 medium (Tholema et al, 1999) containing 10 mM HEPES-NaOH (pH 7.5) or 10 mM MES-NaOH (5.6) with different concentrations of KCl. After overnight growth, plates were observed and photos were taken.

3-4. K^+ content in bacteria

K^+ content in *E. coli* was measured as described before (Matsuda et al 2004; Tsunekawa et al. 2009) with some modification. *E. coli* LB2003 cells harboring the empty vector or CHX23 were cultured in a synthetic medium at 30°C. The cells were collected by centrifugation, suspended in 120 mM Tris-HCl (pH 8.0) and the cell density was adjusted to an OD_{578} of 30. EDTA was added to 1 mM and the cell suspension was shaken for 10 min at 37 ºC, collected by centrifugation, and washed twice with 200 mM HEPES-NaOH at pH 7.5 or 200 mM MES-NaOH at pH 4.3 or pH 5.6, then suspended in the same buffer. After shaking for 20 min at room temperature, cells was adjusted with

the same buffer to an OD_{578} of 3. Ten minutes before starting K^+ uptake measurement, 10 mM glucose was added to the suspension. KCl was added at *time* 0 to a final concentration of 20 mM. At indicated times, one ml of the cell suspension was taken and transferred into a tube containing 150 µl of silicon oil AR200 (SERVA, Heidelberg Germany) and subsequently centrifuged at 12,000 rpm for 1 min. The potassium content of cell pellet was determined by flame photometry with an AAS instrument (Shimadzu Japan). (Experiment performed in Uozumi lab, Japan)

3-5. Microscopy

For the tobacco pollen, fluorescence images were taken under a Nikon E800 microscope (Tokyo, Japan) linked with a charge-coupled device camera (Spot, Diagnostic Instruments, Sterling Heights, MI). For both tobacco and Arabidopsis pollen, confocal images were taken with a Zeiss LSM 510 laser scanning confocal microscope (U. Mass at Amherst for the tobacco pollen). For stably-transformed Arabidosis plants, 10 independent T3 lines were checked and all showed the same GFP signal pattern.

4. RESULTS

A. Determine the membrane localization of CHX23 in pollen

I localized fluorescent-tagged CHX23 in three ways: i) transient expression in leaf protoplast; ii) transient transfection of tobacco pollen, and iii) in Arabidopsis pollen from stably transformed plants driven by its native CHX23 promoter.

A-1 CHX23 was localized on the prevacuolar compartment (PVC) in protoplast

To understand the cellular mechanism of the two CHXs, GFP-tagged CHX23 driven by the CaMV 35S promoter was expressed in isolated leaf mesophyl protoplasts (Fig. III-1). Under confocal microscope, the green signal pattern looked very similar to that from the prevacuolar membrane marker GFP-Syp22 (Fig. III-2), and signals from CHX23-RFP and GFP-Syp22 overlapped well when the protoplasts were cotransfected with these two gene constructs (Fig. III-3). These results indicate that CHX23 was localized on the PVC membrane under those conditions.

A-2 CHX23 was localized on the ER in tobacco pollen

As CHX23 is specifically expressed in pollen (Sze et al., 2004), a male gametophyte with distinct functions from mesophyll cells (Fig. II-1.B), I localized the protein in pollen. Tobacco pollen grains was transfected by bombardment with GFP-tagged to the C-terminal of CHX23 and the construct was expressed using the pollen -specific promoter LAT52. The grains were germinated in vitro and the tubes were examined by microscopy (Fig. III-4 A-G) Markers included the ER protein SIP2.1 (Ishikawa et al., 2005) linked to GFP (Fig. III-4 E) and GFP-HDEL which is an ER lumen retention signal (Fig. III-4 F). When tobacco pollen was co-transfected

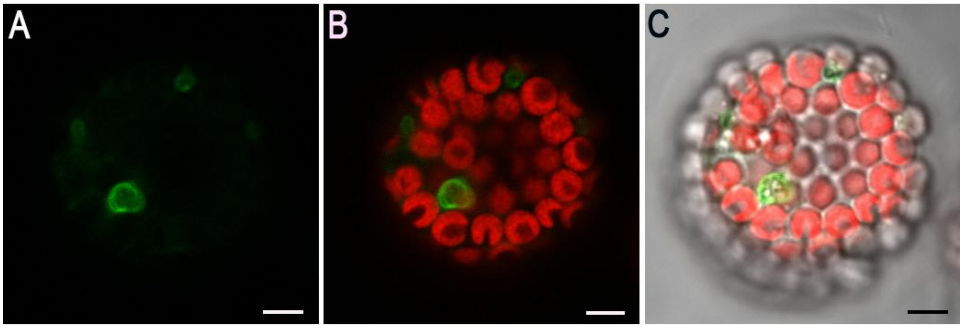
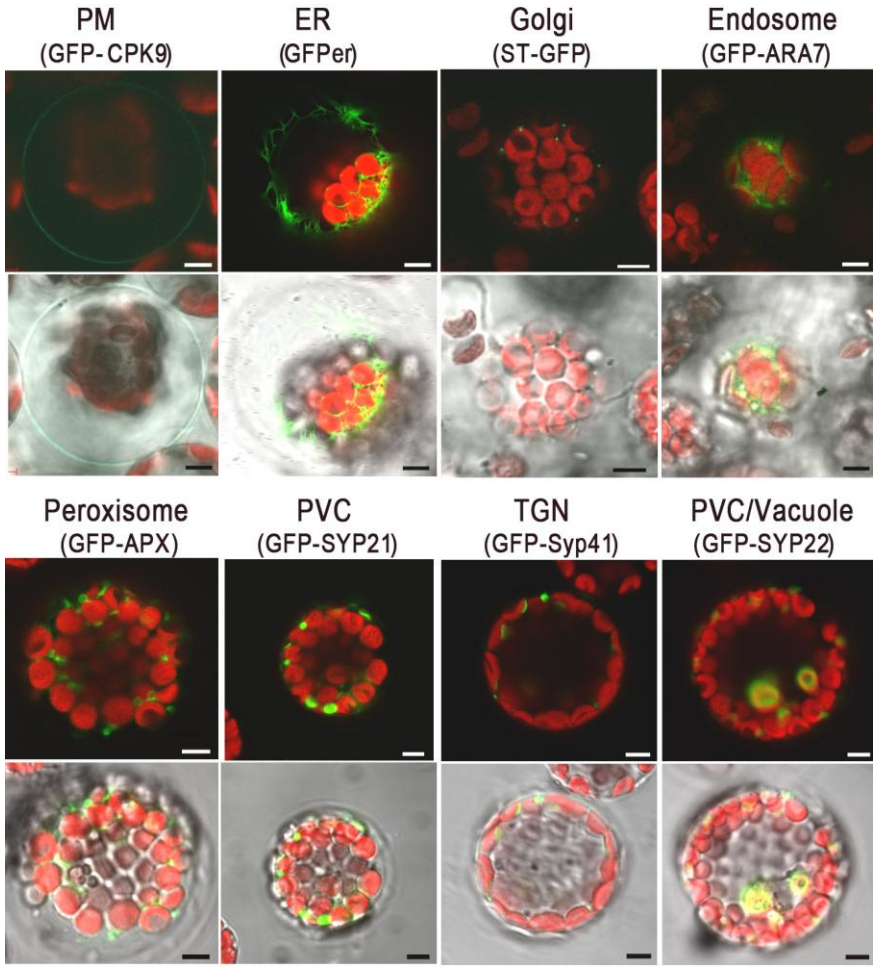


Figure III-1. Membrane localization of CHX23-GFP protein in Arabidopsis protoplasts.

CaMV 35S-driven CHX23 was transiently expressed in isolated leaf mesophyll protoplast. (A) Green signal from CHX23-GFP alone; (B) Overlay of chloroplast-emitted red autofluorescence and CHX23-GFP; and (C) superimposed images of the bright field image, A and B. Images were taken under laser scanning confocal microscope. One representative figure from two independent experiments. Scale, 5 µm.

Figure III-2. Distinct patterns of GFP-linked membrane-markers in Arabidopsis protoplasts.

These constructs were under the CaMV 35S promoter and transiently expressed in isolated leaf mesophyll protoplast. The marker proteins-tagged with GFP are indicated in parenthesis. Top rows show superimposed images of the red and green channel. Chloroplast autofluorescence is red. Lower rows are superimposed images from bright field, and the corresponding top panel with marker-GFP and chloroplast signals. Images were taken under laser scanning confocal microscope. Each figure is from at least two independent experiments. Scale, 5 μm.


PM
(GFP-CPK9)
ER
(GFPer)
Golgi
(ST-GFP)
Endosome
(GFP-ARA7)
Peroxisome
(GFP-APX)
PVC
(GFP-SYP21)
TGN
(GFP-Syp41)
PVC/Vacuole
(GFP-SYP22)

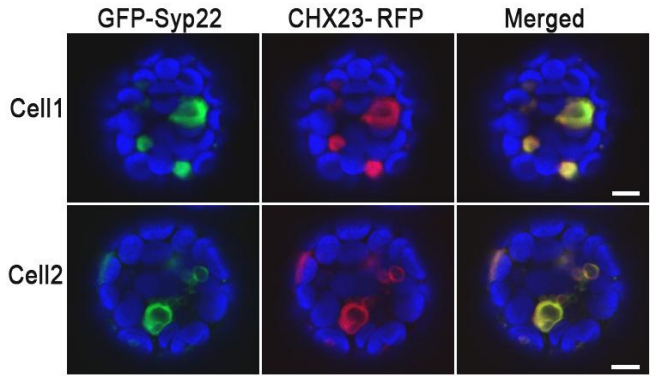


Figure III-3. CHX23 colocalized with GFP-Syp22 a marker of the prevacuolar compartment (PVC) in Arabidopsis protoplasts.

Leaf mesophyll protoplasts were co-transfected with CHX23-RFP and GFP-Syp22 under the CaMV 35S promoter. Images from two representative cells are shown here. Autofluorescnece from chloroplasts were shown in blue. Images were taken under laser scanning cofocal microscope. Representative images from two independent experiments. Scale, 5 µm.

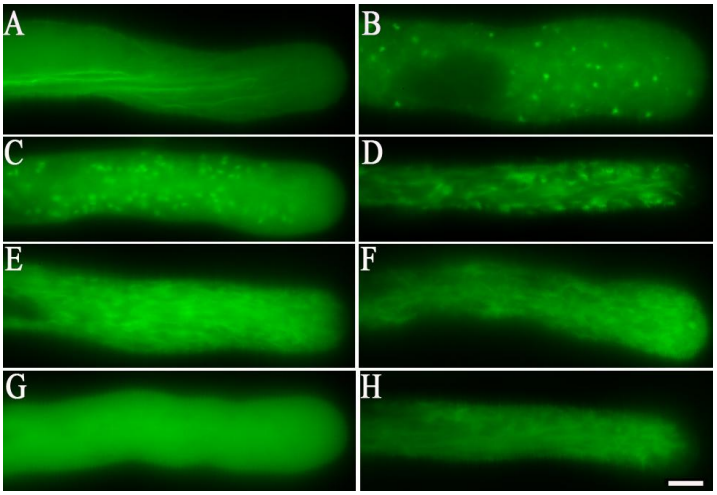


Figure III-4. Patterns of GFP-tagged markers and CHX23 in tobacco pollen tubes.

These constructs had the pollen-specific Lat52 promoter and were transiently expressed in tobacco pollen tubes after bombardment of the pollen grains. Pollen grains were geminated and tube grew in vitro for 4 h at 30 °C before images were taken under fluorescent microscope. Markers and the labeled organelles are: (A), NtPLIM2b-GFP marked actin filament; (B), GFP-Rab5 marked endosome; (C) GFP-Rab2 marked Golgi; (D) Mitochondria-targeted signal peptide-GFP (Cheung AC, unpublished); (E) SIP2.1-GFP marked ER; (F) GFP-HDEL marked ER; (G) Lat52::GFP for cytosol; and (H) CHX23-GFP . Scale, 5 μm.

with CHX23-RFP and SIP2.1-GFP, the red and green reticulate patterns overlapped well (Fig. III-5). Similar results were obtained with GFP-HDEL. These results would indicate that CHX23 was an ER-associated protein.

A-3 CHX23 driven by native promoter was localized to ER-looking membrane in Arabidopsis pollen

To verify whether over-expression might cause mislocalization of membrane proteins, stably transformed plants were generated using CHX23-GFP driven by the CHX23 native promoter. Pollen tubes from T3 Arabidopsis plants germinated in vitro showed similar reticulate pattern (Fig. III-6) as seen in tobacco pollen. These results would confirm the localization of CHX23 to reticulate endomembrane, like the ER in both transiently transfected tobacco pollen and in pollen from stably transformed Arabidopsis plant.

B. Determine the transport activity of CHX23

B-1 CHX23 failed to show activity in yeast cells

To test the transport activity, CHX23 cDNA was cloned and expressed in a yeast mutant KTA40-2 that is defective in cation transporters. However, CHX23 had no effect on KTA40-2 mutant growth (Fig. III-7) or on hygromycin resistance (Fig. III-8), Furthermore, CHX23 did not restore growth of LMM04 mutants (Fig. III-9) that were defective in K^+ uptake (*nha1, ena1-4, kha1, tok1, trk1/2*) as shown for CHX13 (Zhao et al., 2008). To exclude any possibility of autoinhibition from the C tail, C tail-truncated CHX23 was cloned and expressed in the KTA40-2 yeast cells. Still, no effect was found

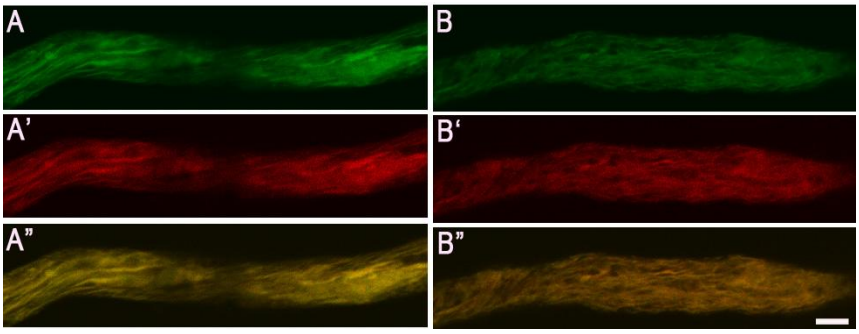


Figure III-5. CHX23 localized on ER in tobacco pollen tubes.

Tobacco pollen grains were co-transfected with GFP-HDEL (A), and CHX23-RFP (A') transiently by bombardment and these genes were expressed with the Lat52 promoter. Pollen was similarly co-transfected with SIP2.1-GFP (B) and CHX23-RFP (B'). Pollen was geminated in vitro and the tube grew for 4 h at 30 °C before images were taken under laser scanning confocal microscope. A" and B" represent merged images for A + A' and B + B', respectively. Scale, 5 μm.

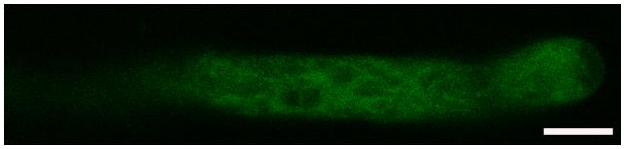


Figure III-6. CHX23 localized to an ER-like membrane in pollen tube of stably transformed Arabidopsis.

CHX23-GFP under its native promoter was stably transferred into Arabidopsis plants. Pollen from the T3 transformants were collected and germinated in vitro for 4 h before images were taken under laser scanning confocal microscope. Pollen tubes from 10 independent T3 lines were checked and all showed the same GFP signal pattern. Scale, 5 μm.

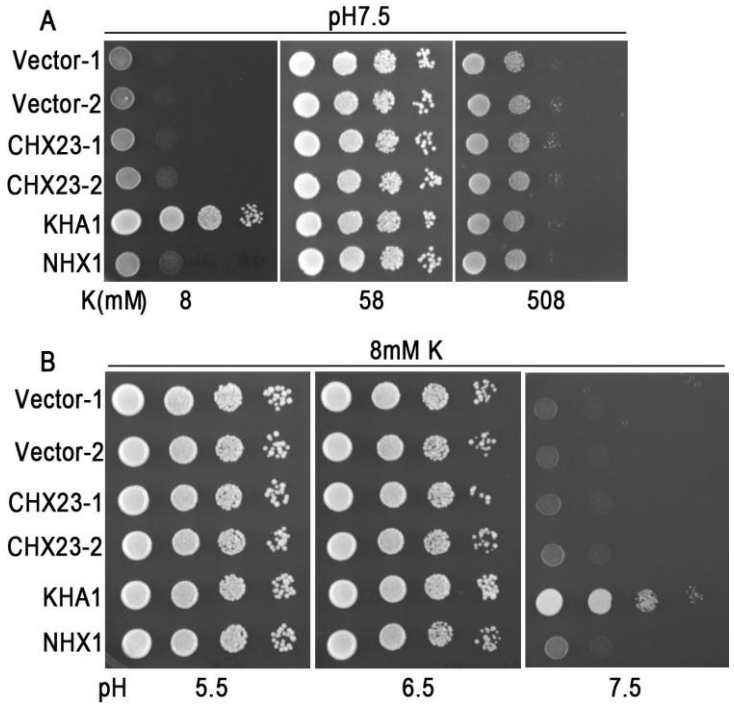


Figure III-7. Expression of full-length CHX23 failed to restore KTA40-2 yeast growth.

KTA40-2 (*ena1-4*Δ *nha1*Δ *nhx1*Δ *kha1*Δ) cells transformed with full-length CHX23 cDNA or empty vector were grown on YNB + MA plates at varied K concentrations and different pH. (A), cell growth at pH 7.5 and different K^+ concentrations; (B), cell growth at 8 mM K^+ but varied pH.. AXT3 (ena1-4Δ nha1Δ nhx1Δ) and LMB01 (ena1-4Δ nha1Δ kha1Δ) cells that contain native KHA1 and NHX1, respectively, served as controls. Images were taken 2 d after growth.

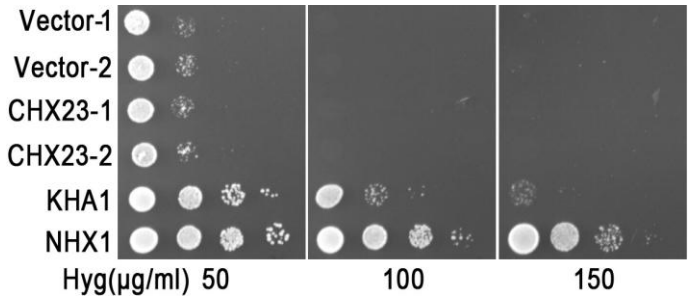


Figure III-8. CHX23 did not confer hygromycin tolerance to KTA40-2 yeast.

KTA40-2 (ena1-4Δ nha1Δ nhx1Δ kha1Δ) cells transformed with CHX23 or empty vector were grown on pH 5.5 YNB + MA plates supplied with different hygromycin concentrations. AXT3 (ena1-4Δ nha1Δ nhx1Δ) or LMB01 (ena1-4Δ nha1Δ kha1Δ) strains that contain native KHA1 or NHX1, respectively, served as positive controls. Images were taken 2 days after growth.

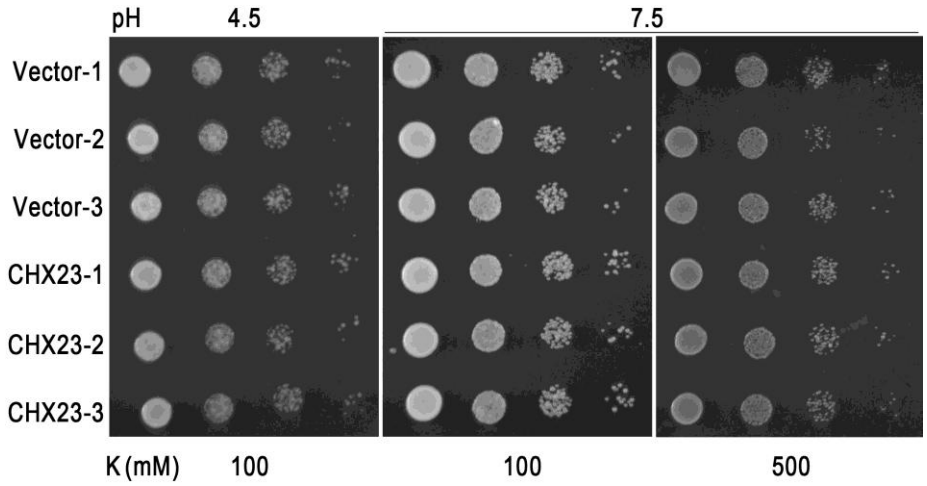


Figure III-9. Expression of full-length CHX23 has no effect on LMM04 yeast growth.

LMM04 (ena1-4Δ nha1Δ trk1-2Δ kha1Δ) cells transformed with CHX23 or empty vector were grown on YNB + MA plates with 100 or 500 mM K at pH 4.5 or 7.5. For each transformant, three different clones were used to do the drop test. Images were taken 2 days after growth.

on cell growth (Fig. III-10). Thus unlike AtCHX20 (Padmanaban et al., 2007) and other closely related homologs, CHX23 showed no activity in yeast.

B-2 CHX23: A role in pH-dependent K^+ transport

To test transport activity in another heterologous system, CHX23 cDNA was cloned and expressed in an E coli mutant (LB2003) which grows poorly on low K^+ medium. CHX23 enhanced bacterial growth of the K^+-uptake defective E coli strain on medium with 10-15 mM K^+ relative to mutants expressing the vector alone (Fig. III-11-A). CHX23 restored growth similar to that of AtKAT1 which codes an inward-rectifying K^+ channel (Nakamura et al., 1995). These results suggest CHX23 has a role in K^+ transport. Using flame photometry, we found that the K^+ content in cells expressing CHX23 increased over time (Fig. III-11-B), indicating that CHX23 mediates K^+ uptake. It is striking that K^+ uptake into E coli is sensitive to external pH. Higher activity is observed at pH 7.5 than at pH 5.6 or 4.3. The results are consistent with the improved growth in CHX23-expressing bacteria at pH 7.5. Though the specific mode of transport is unclear, the results point to a role of CHX23 in K^+ homeostasis in a pH-dependent manner.

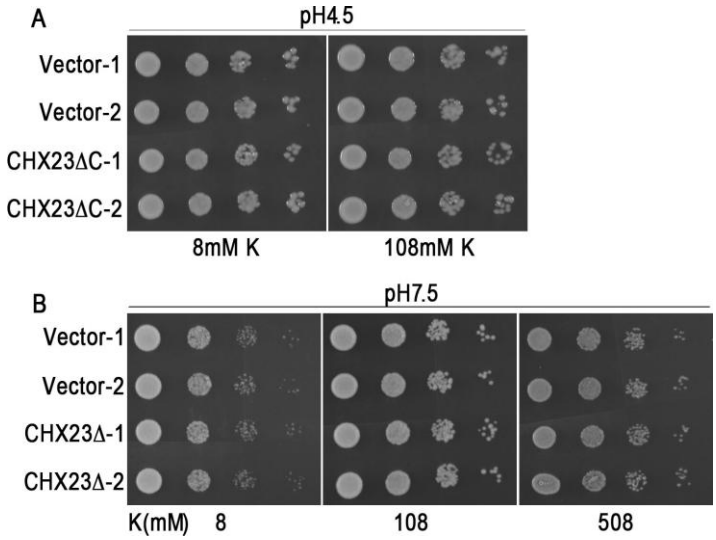


Figure III-10. Expression of C tail-truncated CHX23 has no effect on KTA40-2 yeast cell growth.

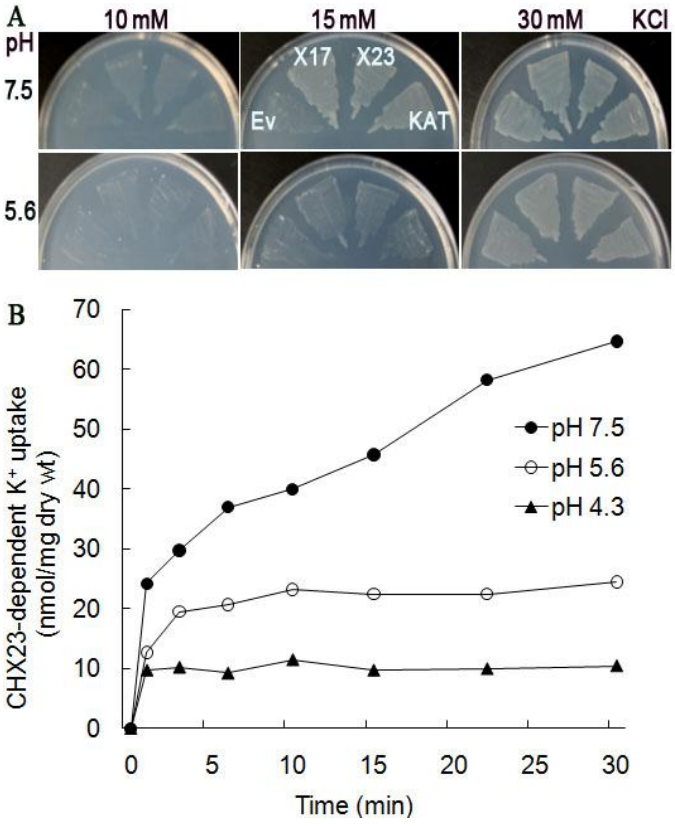
KTA40-2 (ena1-4Δ nha1Δ nhx1Δ kha1Δ) cells transformed with CHX23-447aa-ΔC cDNA or empty vector were grown on YNB + MA plates with different K and pH conditions. (A) Cells grown under the same acidic pH, but different K^+ concentrations; (B) Cells grown under the same alkaline pH, but different K^+ supplies. Images were taken 2 days after growth.

Figure III-11. CHX23 improves bacterial growth by promoting K^+ uptake into K-uptake defective E coli strain LB2003 at pH 7.5.

A. Growth of *E. coli* LB 2003 harboring empty pPAB404 vector, or vector with AtCHX17, $AtCHX23_{779}$ (truncated at C-terminus after Ile_{779}), or KAT1 (K^+ channel) on synthetic solid medium containing 10 mM HEPES-NaOH (pH 7.5) or 10 mM MES-NaOH (5.6) with different concentrations of KCl. Photographs were taken after an overnight incubation at 30°C.

B. Increase in K+ content in *E. coli* LB2003 expressing $AtCHX23_{779}$.

In B, K content assay was performed in 200 mM HEPES-NaOH (pH 7.5) or 200 mM MES-NaOH (pH 5.6 and pH 4.3). Twenty mM of KCl was added into assay buffer. One mL of the cell suspension was taken at the indicated time and transferred into a tube containing 150 µl of silicon oil, and subsequently centrifugated at 12,000 rpm for 1 min. The potassium content of the pellet was determined by flame photometry. The values are averages ±SD from four samples of cell culture.


A
10 mM
15 mM
30 mM
KCl
pH
7.5
5.6
X17
X23
Ev
KAT
B
CHX23-dependent K+ uptake
(nmol/mg dry wt)
Time (min)
pH 7.5
pH 5.6
pH 4.3
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30

5. DISCUSSION

I showed that CHX21 and CHX23 are essential for male fertility and are key players for tube guidance at the funiculus and micropylar stages (Chapter II). *chx21chx23* double mutant pollen failed to target isolated ovules indicating they were incompetent in perceiving and/or responding to ovule cues. To understand the cellular and molecular bases for this phenotype, here I investigated the membrane localization and molecular function of CHX23.

5-1. CHX23 is an ER protein in pollen tube

CHX23 was localized on ER by fluorescence protein-tagging and cotransfection with ER markers linked to GFP in tobacco pollen. Consistently, pollen from Arabidopsis plants transformed with CHX23-GFP driven by the CHX23 native promoter showed similar, though weaker, reticular pattern. It is worthy to note that this native promoter-driven construct was able to complement gene segregation phenotype when transformed to the *chx21-1*/+, *chx23-4/chx23-4* plants, suggesting this construct is biologically functional.

Interestingly, when transiently expressed in isolated mesophyll protoplasts, CHX23-driven by CaM 35S promoter was colocalized to PVC compartments labeled by GFP-Syp22. This discrepancy in CHX23 localization may be due to distinct functions associated with the male gametophyte versus leaf cells. As both CHX23 promoter-driven GUS activity and RT-PCR of pollen RNA showed that CHX23 is specifically expressed in pollen, our studies using tobacco and Arabidopsis pollen suggest that CHX23 is associated with reticulate endomembranes such as the ER.

5-2. CHX23: a role in K^+ and H^+ homeostasis

Based on sequence similarity with known transporters, CHXs were predicted to be cation/proton exchangers (Sze et al., 2004). To investigate the biochemical function, CHX23 was expressed first in yeast mutants that are defective in cation/proton homeostasis regulation. These yeast mutant cells are powerful tools to investigate the functions of many cation transporters. Several vegetative tissue-expressed CHXs have been studied using this heterologous system (Padmanaban et al 2007; Zhao et al, 2008). For unclear reasons, the full-length CHX23 expressed in either KTA40-2 or LMM04 yeast showed no activity compared to vector only transformants. When a putative regulatory region at the C-tail was truncated, CHX23 also failed to show any activity. As the hydrophilic regulatory region can auto-inhibit transport activity, as in ACA-type Ca pumps of Arabidopsis (Sze et al., 2000)**,** the results suggested that failed CHX23 activity in yeast cells was not due to inhibition of the hydrophobic C tail. The CHX23 protein appeared to be expressed based on GFP signals in the transformants, though it may be partially degraded or mislocalized.

Interestingly, expression of CHX23 in *E. coli* strain LB2003, which lacks three K^+ uptake systems (Stumpe and Bakker, 1997), enhanced cell growth under low K^+ and alkaline pH. Compared to empty vector, bacteria transformed with CHX23 showed higher K^+ accumulation at alkaline pH. This suggests that CHX23, like several other CHXs studied, has a role in regulating cellular K^+ and/or H^+ homeostasis, though the transport mechanism is not clear.

Other CHXs functionally characterized also suggest roles in K and pH homeostasis. For example, CHX20, enhanced yeast KTA40-2 cell growth under low K^+ and alkaline pH conditions (Padmanaban et al., 2007). This CHX is specifically expressed in guard cells and localized to a reticulate endomembrane. Homozygous mutant leaves fail to fully open their stomatal pore in response to light, suggesting CHX20 plays an important role in light-induced stomatal opening. CHX17 is preferentially expressed in vegetative tissues, such as roots (Sze et al., 2004), and has been localized to endomembranes in plants. It showed similar function as ScKHA1 (Maresova and Sychrova, 2006) (Chanroj Salil, PhD dissertation), the yeast endomembrane K^+/H^+ exchanger that regulates cation/H^+ homeostasis and enhances cell growth under alkaline pH and low K^+ (Maresova and Sychrova, 2005). Interestingly, expression of CHX13 facilitated LMM04 yeast growth under low K^+ at acidic conditions. $^{86}Rb^+$ uptake assays showed that CHX13 mediated high-affinity K^+ uptake (Zhao et al., 2008). CHX13 is localized to the PM in plants. Although CHXs are expressed in different tissues and have distinct biochemical properties, they share a common trait in their ability to affect K^+ transport at distinct pHs.

5-3. What is the role of ER and CHX23 on polar tip growth and reorientation?

The role of ER in tip growth and in tube reorientation is not clear. ER oscillates in the apical region of pollen tube. Cross-correlation analysis showed that ER oscillates in phase and lead the tube tip growth (Lovy-Wheeler et al., 2007), implying that ER plays a role in tip growth. Iwano et al (2009) used Ca^{2+}-sensitive yellow cameleons to observe Ca^{2+} dynamics in pollen tubes of Arabidopsis and tobacco grown *in vivo* or semi-*in vivo*

assays. Iwano et al (2009) showed that ER is an important Ca^{2+} sink and this Ca^{2+} storage role is important for pollen tube growth. When cyclopiazonic acid, a specific ER-type Ca-ATPase inhibitor, was applied, ER Ca^{2+} was reduced and pollen tube growth inhibited. ER could play an important role in shaping the oscillatory Ca^{2+} gradient at the tip, which was observed in the in vitro growing pollen tubes (Pierson et al., 1996; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997). However, tip Ca^{2+} oscillatory pattern was not found in the *in vivo* and *semi in vivo* growing tubes (Iwano et al., 2009).

The presence of CHX23 at the ER would suggest that ER might contribute to pH dynamics in the pollen tube, in a manner similar to Ca^{2+}. In vitro growing pollen tube shows acidic tip and an alkaline band at the clear zone (Messerli and Robinson, 1998; Feijo et al, 1999). But the biological significance of this H^+ dynamics is not clear.

Several studies suggest that cellular pH has important roles in signaling, such as in root columela cells during gravitropism. Using a cytoplasmic pH-sensitive green fluorescence protein (GFP), Fasano et al (2001) observed a 0.4 unit increase in cellular pH that preceded tropic growth. Altering the cytoplasmic pH with caged protons delayed the gravitropic response. These results suggest that changes of cellular pH form key component of a pathway in the signal perception and/or transduction during root gravitropism (Fasano et al, 2001). In guard cells, ABA treatment led to a 0.04-0.3 unit rise in cellular pH and a 1.5-3 fold increase of cellular Ca^{2+} that precede stomatal closure (Blatt, 2000). Furthermore, IAA exposure resulted in a 1.5-2 fold increase in cellular [Ca], but 0.2-0.4 unit decrease in cellular pH which accompany stomata opening (Irving

et al, 1992). By regulating the activity of the K channels, pH may function as a part of a signaling pathway that is independent of Ca-related events in the ABA response (Blatt, 2000).

I propose that the role of CHX20 in light-induced stomata opening (Padmanaban et al, 2007) is linked to polarized membrane trafficking in the guard cells. The guard cells show structural polarity: i) the cell wall at the mouth of the pore is thick relative to the walls next to the epidermal pavement cells; and ii) the guard cell cytoskeleton radiates away from the pore. This suggests that an increase in cell volume due to turgor pressure might result when exocytosis and insertion of membranes to the PM that faces away from the pore. Functioning as a K^+ and H^+ homeostasis regulator, CHX20 may play a role in determining the axis of the polar exocytosis pathway in guard cell.

Early studies showed that local cellular pH change affects exocytosis. For example, Gluck et al (1982) showed that low pH facilitates exocytosis of membrane containing H^+-ATPase activity in turtle bladder. Certal et al (2008) tested the effect of cellular pH on pollen tube reorientation by adding Gramicidin A to a localized spot next to the tube tip. Gramicidin A, a hydrophobic molecule, forms highly-permeable monovalent cation pore in lipid membranes thus exchanging K^+ efflux for H^+ uptake. The resulting local cellular acidification caused tube reorientation toward the site of the added ionophore (Certal et al, 2008). These results would support the idea that localized pH change constitutes a key part of a signaling pathway that regulates directional tube polar growth.

5-4. Models of CHX21 and CHX23 in pollen tube guidance

To reorient the axis of polarity in pollen tubes, it is necessary to shift the machinery for polar tip growth to a new growth site. This change would include shifts in tip-focused Ca^{2+} gradient, organization of actin filaments, Rop GTPase activity, and the site of exocytosis. How would a K^+/H^+ exchanger participate in this process in response to guiding signal (s) from ovule/embryo sac? Based on the current literature and knowledge, I propose two models here for the function of CHX21 and CHX23 in pollen tube guidance: Guiding molecule(s), such as peptides secreted from the ovule/embryo sac, are sensed by unidentified PM receptor(s) that initiates a signaling pathway near the PM site where the concentration of the cue is highest. This signaling pathway could activate ER-localized CHX21 & CHX23. As ER oscillates into the apical region, the activity of CHX23/CHX21 could change 1) localized cellular pH and/or 2) the electric current pattern observed in cells undergoing development, thus shifting the axis of polar growth.

5-4-1. Local cellular pH change

Such pH shifts could regulate one or more of the following processes (Fig. III-12).

Shift in tip-focused Ca dynamics at the tip

This microdomain cellular pH change activate local PM Ca^{2+} channel, which results in cellular Ca^{2+} increase due to Ca^{2+} influx and tube reorientation toward the direction the guiding signal is perceived.

Malho and Trewavas (1996) found that modification of apical cellular Ca^{2+} induces tube bending toward the side of higher Ca^{2+}. It is possible that voltage-gated Ca^{2+} channels in pollen (Malho et al., 1995; Shang et al., 2005; Wang et al., 2004) are

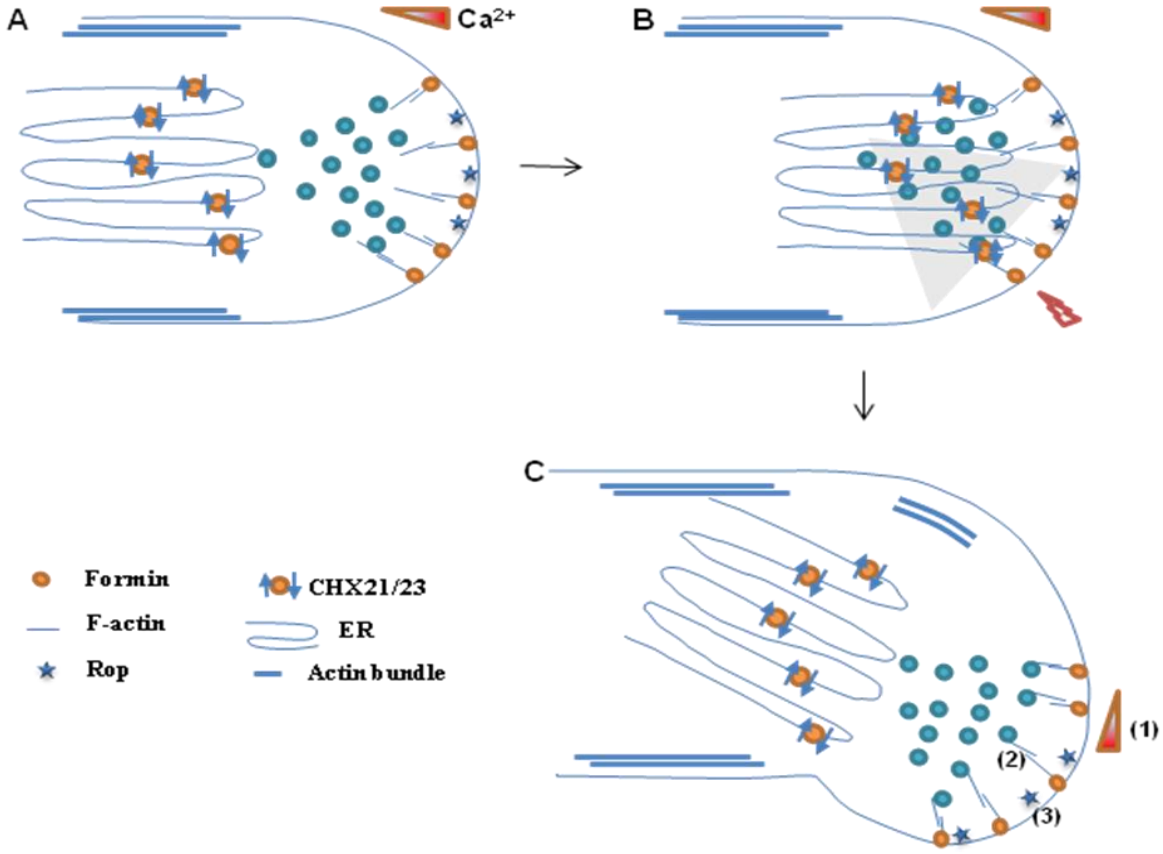


Figure III-12. Model showing roles of CHX21 and CHX23 in pollen tube guidance.

(A) Tip growth in pollen tube. (B-C) Model of shift in polarity. Upon receiving a guiding signal, CHX21 or CHX23 is activated and changes local cellular pH in the apical region. In (C), by changing tip-focused Ca^{2+} gradient (1), actin filament (2), or relocating Rop activity (3), CHX21/23 plays a critical role in altering a signaling network that sets up a new growth axis directed toward the site where the guiding signal was perceived.

activated by the pH changes. Interestingly, cAMP was shown to also induce pollen tube reorientation. Loading cAMP near the tip of an in vitro growing tube or manipulating cellular cAMP by photo activating caged cAMP both induced tube turning toward the direction of higher cAMP (Moutinho et al., 2001). Possibly, by opening cAMP-gated Ca^{2+} channel, cAMP increased local cellular Ca^{2+} which induce tube turning. In supporting this idea, cAMP has been shown to induce shifting of growth direction of the neuron cell in a Ca^{2+}-interconnected manner (Song et al., 1997; Petersen et al., 2000).

It is possible that local increase of cellular Ca^{2+} shift the polar actin "track" toward the site of guiding signal perception. Thus modifying the activity of actin-binding proteins would change vesicle delivery along the actin filaments toward this site. By setting up a positive feed-back loop, a new growth site is established.

Actin filament dynamics

Local pH changes may regulate actin filament dynamics through modification of actin-binding proteins, independent of the Ca^{2+} pathway. Actin filament dynamics is pH-dependent. Actin filaments from pea and rice root cells depolymerized fast at pH 8.0 or above, but slowed down in neutral or slightly acidic pH in vitro (Andersland and Parthasarathy, 1993). This pH-dependence may attribute to ADF/cofilin , which severes actin and is pH-sensitive. In supporting this idea, NHE1, a PM Na^+/H^+ exchanger, was found to be critical for actin polymerization in attaining Dictyostelium polarity for cell motility (Patel and Barber, 2005). In *nhe1* mutant cells, less free barbed ends of actin filament was observed due to lower local cellular pH caused by defective NHE1 (Frantz et al., 2008). Changes of local cellular pH caused by CHX21/23 activity may shift the polar actin "track" for the original tip growth in response to perception of the guiding

signal at another site. As a result, new growth site is set and tube reorients toward the direction of the guiding signal.

Reorganization of Rop1 to a new site

Rop1 is thought to be a 'master' regulator of polar tip growth, and its activity can be switched by RopGEF. Studies showed that PM-localized receptor-like kinase (RLK) can bind to RopGEF (Kaothien et al., 2005; Zhang and McCormick, 2007), an activator of the pollen Rop1 small GTPase (Lee and Yang, 2008). I propose that the local cellular pH changes may favor the binding or dissociation between RopGEF (s) and RLK (s). Upon binding of guiding cue(s), this RLK (s) get activated. Activated RLK would bind and activate RopGEF, which in turn produces polar Rop activity at that particular location. By regulating actin dynamics through two counteracting pathways (Gu et al., 2005), ROP1 mediates vesicle delivery and fusion at the apical plasma membrane (Lee and Yang, 2008). Thus, Rop activity at a new site could organize a new polar axis and shift the growth direction towards the ovule/embryo sac.

5-4-2. CHX23/CHX21 shifts the pattern of electric currents

Polarized pattern of electric currents have been detected in developing cells and organisms in which cations such as H^+, K^+ or Ca^{2+} enters the growing tip (Harold, 1986). Thus local ion flux or gradients were proposed to trigger development. Another model is that CHX23/CHX21 activity has a role in resetting a new current pattern in response to ovule cues, and so establish a new site for tip growth in the pollen tube (Hepler et al, 2006). It is possible polarity results from redistribution of transport proteins, however this author is inclined to favor the model that localized ion and H^+ flux act as informational signals to establish a new site of polar tip growth.

IV. CONCLUSION AND FUTURE DIRECTIONS

1. CONCLUSIONS

This study identified two cation/proton exchangers as essential molecules in pollen tube guidance. The novel finding from this study contributes to a better understanding about the fundamental mechanisms of polar tip growth in eukaryote cells in general, and highlights the biological significance of similar cation/proton exchangers in plants.

1-1. How does this study contribute to the understanding of polar cell growth?

Polarized growth is fundamental for organisms across kingdoms in order to develop and survive. Examples include the pollen tube and root hair in plants, hyphae in fungi and neuron cells in animals.

In flowering plants, pollen tube functions as a sperm delivery tool and is crucial for reproduction and species preservation. In the soil, root hair growth is essential for the plants to adapt to and survive on various soil conditions. For example, in response to low phosphorus, plants grow more and longer root hairs in order to extend the “exploring” area and reach more nutrients. Similarly, hypha growth is critical for fungus to invade host cells and get nutrients. In animals, neuron cells are the core components of the nerve systems and are essential for development and responses to the environment. Extensive studies in these cells identified similar core cellular and molecular components for the tip growth, like the tip-focused polar exocytosis apparatus, Ca^{2+}, cytoskeletons and the Rho family small GTPase. However, fundamental questions remain unanswered, like in response to changing environments, how do these polarized growing cells reorient and

change growth direction? When polar growing cells reorient, the original polar vesicle delivery axis will need to dissipate and a new delivery axis is established. How cells reorganize the key molecules for tip growth temporally and spatially during this "shifting" process is poorly understood.

In Xenopus spinal neurons growing in culture, cAMP was shown to induce shifting of growth direction of the neuron cell (Song et al., 1997), possibly by opening up PM Ca^{2+} channels and increasing local cellular Ca^{2+} (Petersen et al., 2000). But what are the direct effectors of cellular Ca^{2+} changes, and how changes of these effectors are temporally organized are not known. In *Dictyostelium discoideum*, a slime mold that adopts polarized cell movement in chemotaxis, Patel and Barber (2005) showed that NHE1, a PM Na^+/H^+ exchanger, was critical for actin polymerization in attaining polarity for cell motility. In *nhe1* mutant cells, less free barbed ends of actin filament was observed due to lower local cellular pH (Frantz et al., 2008). These results point to a role of local cellular pH in cell polarity.

My study identified two cation/proton exchangers as key players for pollen tube turning in response to guiding signal (s) from ovule/embryo sac. To my knowledge, these are the first identified transporters that function in reorientation of polarized tip growth in eukaryotes. This adds to the list of key molecules involved in polarized cell growth, and will facilitate understanding the mechanism of polar cell growth in other polar growing cells in eukaryotes.

1-2. How does this study contribute to the understanding of cation/proton exchangers?

Several cation/proton exchangers from plants and yeast have been implicated in conferring salt tolerance and in regulating pH homeostasis. SOS1 is a PM-localized Na^+/H^+ antiporter that has a role in salt tolerance (Shi et al., 2002). Possibly by using energy from H^+ gradient created by the PM H^+-ATPase, SOS1 transport Na^+ out of the cell. NHX1 is a vacuole-localized protein that also confers salt tolerance in plants, by transferring Na^+ from cytosol to vacuole (Apse et al., 2003). Interestingly, NHX1 also regulate cellular and luminal pH. In the wild-type Japanese morning glory, petals are bright blue color due to alkaline pH inside vacuole. Mutation of NHX1 resulted in failed pH regulation in the vacuole and a lighter petal color (Fukada-Tanaka et al., 2000). Similarly, NHX1 homolog in yeast enhanced cell salt tolerance by sequestrating Na^+ into vacuole (Nass et al., 1997), and regulated cellular and luminal pH. *nhx1* mutant was more sensitive to low pH, showed acidified vacuolar lumen and membrane (Brett et al., 2005) trafficking defect .

The biological implications of several vegetative tissue-expressed CHXs have been studied. For example, CHX13 is a PM-localized transporter that mediate high-affinity K^+ uptake (Zhao et al., 2008). T-DNA insertion mutants were sensitive to lower K^+ and growth was retarded. CHX17 was shown to play a role in K^+ uptake in plant, but the mechanism is not clear (Cellier et al., 2004). These studies suggest that cation/proton exchangers could play roles in plant nutrition and K^+ homeostasis.

Padmanaban et al (2007) showed that CHX20 plays a role in light-induced guard cell movement, possibly by regulating K^+/H^+ homeostasis. Considering the possible

polarity of guard cells during stomatal opening, CHX20 may participate in the regulation of polar vesicle delivery to the PM to increase its area. In my study, results suggest that CHX21 and CHX23 play essential role (s) in pollen tube reorientation in response to guiding signal (s) from ovule/embryo sac. These studies suggest that by manipulating microdomain cellular pH and/or K^+, cation/proton exchangers may play essential roles in cellular signaling.

1-3. Why does pollen need so many CHXs?

Among 28 CHXs, about 20 are expressed in pollen. Why would a simple *gametophyte* with only three cells need so many CHXs? This question may look less confusing when the complexity of pollen development and function are considered.

During pollen development, there are several distinct steps, include two consecutive meiosis, which produce four microspores from one pollen mother cells, and two mitosis steps that produce two sperm cells and one vegetative cell (Fig. I-1). Microgametogenesis involves cell growth, division and differentiation. After pollen lands on a stigma, events will occur before pollen germinates and protrudes a tube. These include recognition between pollen and stigma cells, hydration, polar growth site initiation and maintenance. Then pollen tube penetrates and interacts with different female tissues and finally reaches the ovule (Fig. I-3). After reception, pollen tube discharges and releases the sperm cells, which will then migrate and fuse with egg and central cells, respectively. By regulating cellular/luminal pH, different CHXs may participate in one or more steps in pollen development, germination, tube growth and guidance and gamete fusion, and the function may be stage-specific. In supporting this

idea, transcriptome data showed that expression of CHXs differs during pollen development (Honys and Twell, 2004), pollen germination and tube growth (Wang et al., 2008; Qin et al., 2009) and after interaction of pollen with pistil cells (Qin et al., 2009). Intriguingly, expression of CHXs was also found in sperm cells (Borges et al., 2008).

In addition to the expanding plasma membrane, growing pollen tubes have very dynamic endomembrane system, like ER, vacuole, and the tip-focused small vesicles (Hicks et al., 2004; de Graaf et al., 2005; Lovy-Wheeler et al., 2007). CHXs may be localized on different membranes. Furthermore, these CHXs may be regulated by different mechanisms and have different affinities. Studies of several CHXs in yeast support this hypothesis (Chanroj S, unpublished results). For example, CHX20 showed K^+ transport activity at alkaline pH, while CHX13 transport K^+ at acidic pH (Padmanaban et al 2007; Zhao et al 2008).

Homologs of CHXs are found in the other plant species , including the monocot rice (*Oryza sativa*) (Sze et al., 2004; International Rice Genome Project, 2005) and maize (*Zea mays*) (Aramemnon, http://aramemnon.botanik.uni-koeln.de/), and the dicots, grape (*Vitis vinifera*) (Jaillon et al., 2007), Medicago truncalata (Aramemnon DB) and black cotton wood (*Populus trichocarpa*) (Tuskan et al., 2006). Possibly, the role of CHXs in plant reproduction is conserved across plant species.

2. FUTURE PROSPECTS

After this study, key questions remaining unanswered are: 1) Is local cellular pH changed by CHX21 and CHX23? 2) How are the activities of CHX21 and CHX23 regulated? 3) Are CHX23 and CHX21 involved in signaling sensing/interpretation or

response in pollen tube guidance? (Is the machinery for reorientation still intact and functional in the double mutants?). Hypothesis and approaches to answer these questions are listed below:

2-1 Is local cellular pH changed by CHX21 and CHX23?

One hypothesis from my studies is that CHX21 and CHX23 may change local cellular pH. One approach to test this idea is to transfer pH-sensitive green fluorescence protein (GFP) (Miesenbock et al., 1998) into pollen, and then use the semi *in vivo* system to test if there are differences in local cellular pH changes between *chx21chx23* and Wt pollen in the presence of ovule. If the activities of CHX23 and CHX21 cause local cellular pH changes, fluorescence signals from Wt and the mutant pollen tubes would be different in the presence of ovule. A complementary experiment is to microinject caged-H^+ into growing *chx21chx23* pollen tubes. If the mutant tube reorients upon photolysis to release the pH reporter, that implies that CHX23 and CHX21 change local cellular pH, which in turn lead to tube reorientation.

2-2 How are the activities of CHX23 and CHX21 proteins regulated?

One of my hypotheses is that CHX23 and CHX21 gets activated after pollen perceives the guidance signal(s) from the ovule/embryo sac. If so, I expect that CHX23 might physically interact with other proteins that modify CHX23, including protein kinases or phosphatases. To test this, one could i) look for interacting partners, and ii) test if CHX23 is modified by phosphorylation. So, two specific questions can be addressed here:

What proteins interact with CHX23 and CHX21?

A split-ubiquitin membrane yeast two hybrid (Y2H) screen (Iyer et al., 2005) can be carried out to search for CHX23 protein interacting protein (s). To do this, a cDNA library from pollen tubes growing semi *in vivo* need to be constructed. Other protein-protein interaction assays can be done to confirm and/or complement the candidate molecules from the Y2H screen, like Biomolecular Fluorescence Complementation (BiFC) and/or coimmunoprecipitation (Co-IP).

Is CHX23/21 modified by phosphorylation?

To test this hypothesis, proteins from Wt pollen tubes growing semi *in vivo* with or without the presence of ovules can be isolated. Next, phosphorylation status of the two protein samples can be tested by immunoblot. The same protein with different phosporylation status detected by SDS-PAGE containing polyacrylamide-bound Mn^{2+}-Phos-tag (Kinoshita et al., 2006). The CHX21-specific antibody is available (Hall et al., 2006). The results of this part can complement the interaction partner screen experiment.

2-3 Is CHX23/21 involved in signaling sensing/interpretation or response?

(Is the machinery for reorientation still intact and functional in the double mutants?)

The hypothesis is that *chx21chx23* double mutant pollen maintains the ability and cellular and molecular components to reorient, however the sensing/interpretation of the guidance cues failed. To test this hypothesis, reorientation of Wt vs. *chx21chx23* pollen tubes growing semi *in vivo* could be tested by local manipulation of Ca^{2+} or cAMP on growth medium. Malho and Trewavas (1996) showed that locally applied Ca^{2+} close to

the tip changed pollen growth direction toward higher Ca^{2+} side. Similarly, pollen turned to the side on medium with locally applied cAMP (Moutinho et al., 2001). If the "turning machinery" is functional, *chx21chx23 pollen* would change direction like Wt in the presence of locally added Ca^{2+} or cAMP, which would delineate that CHX23 and CHX21 play a role in signaling sensing/interpretation. If the *chx21chx23* pollen fails to turn, that would suggest that the "turning machinery" is broken in the mutant pollen and that CHX23 and CHX21 plays a role in transducing the signaling response.

V. APPENDICES

A. Materials

1. Wild type & mutant plant lines used in this study

Wt	Mutant name	Identification no/ description	Ecotype	Marker	Intron/exon	Source
	chx21-2	SALK_085865	Col	Kan	Exon	ABRC
	chx21-s1	SAIL_162_A07	Col	Basta	Exon	ABRC
	chx21-s2	SAIL_154_E09	Col	Basta	Exon	ABRC
	chx23-4	SALK_035909	Col	Kan	Exon	ABRC
Wt		Wt, ecotype Col-0	Col			ABRC
Wt_i	Imp-1 (aos)	Enhancer trap At5G42650	Col			Dr. Z. Liu
Wt_{GUS}		pLat52 :: GUS	Col	Basta		Dr. Ravi Palanevilu

2. Transgenic plants generated in this study (CHX23p::CHX23-GFP)

Line	HM	Plant background	Marker	Construct	Source
CHX23GFP-1-5	Yes	Wt Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu
CHX23GFP-3-2	Yes	Wt Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu
CHX23GFP-4-1	Yes	Wt Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu
CHX23GFP-6-8	Yes	Wt Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu
CHX23GFP-12-4	Yes	Wt Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu
CHX23GFP-12-5	Yes	Wt Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu
CHX23GFP-14-4	Yes	Wt Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu
CHX23GFP-25-11*	Yes	*chx21*$^{+/-}$*chx23*$^{-/-}$ Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu
CHX23GFP-45-26*	Yes	*chx21*$^{+/-}$*chx23*$^{-/-}$ Col	Hygromycin	CHX23p::CHX23-GFP	Y.X Lu

3. Yeast strains used in this study

Strain Name	Genotype	References
	A. Yeast cells	
KTA40-2	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mall0 ena1*D::*HIS3*::*ena4*D *nha1*D::*LEU2 nhx1*D::*TRP1 kha1*D::*kanMX*	Maresova and Sychrova, 2005
LMM04	*Mata ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mall0 ena1*D::*HIS3*::*ena4*D *nha1*D::*LEU2 trk1*D::*LEU2 tra2*D::*HIS3 kha1*D::*kanMX*:*tok1*D	Maresova and Sychrova, 2005

AXT3	Δ*ena1-4::HIS3* Δ*nha1::LEU2* Δ*nhx1::TRP1*	Quintero et al, 2000
LMB01	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mall0 ena1*D*::HIS3::ena4*D *nha1*D*::LEU2 kha1*D*::kanMX*	Maresova and Sychrova, 2005

4. Yeast transformants

Strain name	Yeast background	Plasmid	Seletion Marker	promoter
CHX23-YESDR196/KTA40-2	KTA40-2	pYESDR196-X23	-ura	PMA1
CHX23-GWFDR196/KTA40-2	KTA40-2	pGWFDR196-X23	-ura	PMA1
CHX23-447aa-ΔC-YESDR196/KTA40-2	KTA40-2	pYESDR196-X23-447aa-ΔC	-ura	PMA1
CHX23-447aa-ΔC-GWFDR196/KTA40-2	KTA40-2	pGWFDR196-X23-447aa-ΔC	-ura	PMA1
CHX23-YESDR196/ LMM04	LMM04	pYESDR196-X23	-ura	PMA1
CHX23-GWFDR196/ LMM04	LMM04	pGWFDR196-X23	-ura	PMA1

5. Bacterial strain used in this study (in Uozumi lab)

Strain Name	Genotype	References
E. coli LB2003	(F$^-$ *kup1* Δ*kdpABC5* Δ*trkA rpsL metE thi rha gal*	(Stumpe and Bakker, 1997)

6. Vectors used in this study

Vectors	Description	Source
Conventional cloning		
pLatGWF7	Lat52-driven GFP high copy vector, for pollen transient expression	YX Lu (GY.Wang)
pLatGWR7	Lat52-driven RFP high copy vector, for pollen transient expression	YX Lu (GY.Wang)
pPAB404-X23	E.Coli expression vector (pPAB404 got from Uozumi lab)	YX Lu
Gateway cloning		
Entry clones		
pECHX23_110807	CHX23 cDNA sequence (c tail in frame) inserted into pDNOR221	YX Lu
pECHX23 pro+cod-stp	CHX23 genomic region, including the promoter region and the ORF (stop codon removed)	YX Lu
Expression clones		
pYESDR196-CHX23	PMA1::CHX23, expression in yeast	YX Lu
pYESDR196-X23-447aa-ΔC	PMA1::CHX23-447aa-ΔC, expression in yeast	YX Lu
pGWFDR196CHX23	PMA1::CHX23-GFP, expression in yeast	YX Lu
pGWFDR196-X23-447aa-ΔC	PMA1::CHX23-447aa-ΔC-GFP, expression in yeast	YX Lu

P2GWF-X23	High copy, 35S::CHX23-GFP, expression in protoplast	YX Lu
P2GWR-X23	High copy, 35S::CHX23-RFP, expression in protoplast	YX Lu
pLatGWF-X23	High copy, Lat52::CHX23-GFP, expression in pollen	YX Lu
pLatGWR-X23	High copy, Lat52::CHX23-RFP, expression in pollen	YX Lu
pMDC107-X23	Binary, CHX23p::CHX23-GFP, expression in pollen	YX Lu
GFP-tagged membrane markers		
Expression in protoplast		
GFP-CPK9	PM marker, P(35S)::TAP-GFP-CPK9	J-Y Lee & JF Harper
GFP-TIP	Vacuole marker, P(35S)::TAP-GFP-TIP	J-Y Lee & JF Harper
GFP-APX		J-Y Lee & JF Harper
GFPer	ER marker, P(35S)::spGFP-HDEL	I Hara-Nishimura
ST-GFP	trans-Golgi marker, P(35S)::ST-GFP	I Hwang
GFP-ARA7	Endosome marker, p(35S)::GFP-ARA7	Ueda T
GFP-APX	Peroxisomes marker, p(35S)::GFP-APX	Harper JF
GFP-SYP21	PVC marker, p(35S)::GFP-SYP21	Ueda T
GFP-SYP22	PVC/Vacuole, p(35S)::GFP-SYP22	Ueda T
GFP-SYP41	TGN marker, p(35S)::GFP-SYP41	Ueda T
Expression in pollen		
NtPLIM2b-GFP	Actin marker, p(Lat52)::NtPLIM2b-GFP	Cheung A.
GFP-Rab5	Endosome marker, p(Lat52)::GFP-Rab5	Cheung A.
GFP-Rab2	Golgi marker, p(Lat52)::GFP-Rab2	Cheung A.
Mito-GFP	Mitochondria marker, p(Lat52)::Mito-GFP	Cheung A.
SIP2.1-GFP	ER marker, p(Lat52)::SIP2.1-GFP	Cheung A.
GFP-HDEL	ER marker, p(Lat52)::GFP-HDEL	Cheung A.
Lat52::GFP	Cytosol, p(Lat52)::GFP	Cheung A.

7. Primers used in this study. Bold sequences are enzyme sites

Purpose	Primers	Primer Sequences
cDNA Cloning	CHX23Cf	5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC** TCCGACCAGCCATCGTAGGA-3'
	CHX23Cr	5'-**GGGGACCACTTTGTACAAGAAAGCTGGGTC** ATCCTCTTCATCATCTTCATCTTCG-3'
CHX23ΔC cDNA cloning	CHX23CΔf	5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC** TCCGACCAGCCATCGTAGGA-3'
	CHX23CΔr	5'-**GGGGACCACTTTGTACAAGAAAGCTGG** GTATGCAAACGCGAGAAGAGGCTC-3'
pPAB404-CHX23 cloning	AtCHX23-BglII-SE	5'-**GAGAGATCT**ATGTCTTCCGGAGCCCC-3'
	AtCHX23-XhoI-AN	5'-**ATTCTCGAG**TTACCTATGAATTCCATATTGATG-3'
CHX23 genomic DNA cloning	CHX23GCf	5'-**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC** GCTACACTCCTAGATCAGAGTAAACAA-3'
	CHX23GCr	5'-**GGGGACCACTTTGTACAAGAAAGCTGGGTC** CCTATGAATTCCATATTGATGATCC-3'
Lat52 promoter cloning	Lat52 Pf	5'-**CGAGCTC** ACTCGACTCAGAAGGTATTGAGGA-3'
	Lat52 Pr	5'-**GGACTAGT** TTTAAATTGGAATTTTTTTTTTTGGTG-3'
chx21-2 RT-PCR	X21RT-F3	5'-AGAGCTGATATTGGTTACATGAAC-3'
	X21RT-R3	5'-GATTCAAACCGGACAGTCGT-3'
	X21RT-F4	5'-GGAGAGTCAGAGCTTTGTGTATTGA-3'
	X21RT-R4	5'-TGAGGTCACAGGTGTTTCTACTTGA-3'
	X21RT-F5	5'-TGACTTATGTAGAGAAAGTGGTTA-3'
	X21RT-R5	5'-TCATCTGCGCATACTGTACTGA-3'
chx21-s1 RT-PCR	X21RT-F1	5'-CTGAATCTATTGATGCAAGTTCGT-3'
	X21RT-R1	5'- GAAAGGAGGAAGACATAGAGGCTTA-3'
	X21RT-F2	5'-CAACTGTCTGTCGCTAATCTTACCT-3'
	X21RT-R2	5'-AGAATCCAAGTGCACAAATCAGTAAC-3'
	X21RT-F4	5'-GGAGAGTCAGAGCTTTGTGTATTGA-3'
	X21RT-R4	5'-TGAGGTCACAGGTGTTTCTACTTGA-3'
chx23-4 RT-PCR	X23RT-F1	5'-TAGCCTCTTCCCTTCCGTTC-3'
	X23RT-R1	5'-CACAAATGATGTAGAAGAGAGGCA-3'
	X23RT-F2	5'-TGGTCTGTCGCGCTAGCCT-3'
	X23RT-R2	5'-ACTAGAAGCGCGATCGTCATATGAGTAT-3'
	X23RT-F3	5'-CGTGGTGAATCAATGAAAAGAGAAGT-3'
	X23RT-R3	5'-ACATAGACTTCATAAATGGCTCATGA-3'
Actin11 RT-PCR	Actin-S	5'-ATGGCAGATGGTGAAGACATTCAG-3'
	Actin-AS	5'-GAAGCACTTCCTGTGGACTATTGA-3'

chx21-2 PCR	X21-2-LP	5’-TTTTTGCTATCCACCTAGTCGAGCTAAC-3’
	X21-2-RP	5’-CGGTTTGTCTGTTAGCAGAAGAGTATTGT-3’
	LBa1	5’-TGGTTCACGTAGTGGGCCATCG-3’
chx21-s1 PCR	X21-S1-LP	5’-GTTTAAATCTATTAAGGAAAATCCCCC-3’
	X21-S1-RP	5’-AGAATCCAAGTGCACAAATCAGTAAC-3’
	pCSA110-LB3	5’-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3’
chx23-4 PCR	X23-4-RP	5’-ATCAGGTAAAGATTCCTACAATTAGTTCA-3’
	X23-4-LP	5’-ACTAGAAGCGCGATCGTCATATGAGTAT-3’
	LBa1	5’-TGGTTCACGTAGTGGGCCATCG-3’
CHX23-GFP trans-gene	X23G-F	5’-GATCCTTATGCCTCTCTTCTACATC-3’
	X23G-R	5’-CTAATTCAACAAGAATTGGGACAAC-3’
CHX23 cDNA Sequencing	X23CS1	5’-TAGCCTCTTCCCTTCCGTTC-3’
	X23CS2	5’-CACAAATGATGTAGAAGAGAGGCA-3’
	X23CS3	5’-ACTAGAAGCGCGATCGTCATATGAGTAT-3’
	X23CS4	5’-TCTGCTCTGACTCTGTCCGA-3’
	X23CS5	5’-GGATGAGATTAGAGCTTCGC-3’
	X23CS6	5’-ACATAGACTTCATAAATGGCTCATGA-3’
g CHX23-GFP Sequencing	X23GS1	5’-CTTGAGGAATCTAAAAATTGCTTCA-3’
	X23GS2	5’-GCAGATTTTGTAACCGTACAATTAGA-3’
	X23GS3	5’-TAGCCTCTTCCCTTCCGTTC-3’
	X23GS4	5’-CACAAATGATGTAGAAGAGAGGCA-3’
	X23GS5	5’-ACTAGAAGCGCGATCGTCATATGAGTAT-3’
	X23GS6	5’-TCTGCTCTGACTCTGTCCGA-3’
	X23GS7	5’-GGATGAGATTAGAGCTTCGC-3’
Lat52 promoter sequencing	Lat52 Pf	5’-**CGAGCTC** ACTCGACTCAGAAGGTATTGAGGA-3’
	Lat52 Pr	5’-**GGACTAGT** TTTAAATTGGAATTTTTTTTTTTGGTG-3’

B. Methods

1. Pollen Assays

1-a. *In vitro* pollen germination

Aim and rationale

Test whether wild-type and single mutant pollen differed in their tube growth in vitro.

Material: Plant growth

Wild-type Arabidopsis thaliana (ecotype Col-0), mutants, and transgenic plants were grown in Miracle-Gro potting soil (Scotts) under well-controlled environmental conditions at 21 ºC, 150 µE m^{-2} s^{-1}illumination for a 16 h photoperiod at 55% humidity.

Germination medium was the same as that used by Fan et al (Fan et al., 2001).

Table A-1-1. Pollen germination medium:

Chemical	Stock conc. (mM)	Final conc. (mM)	V stock/40 ml
MES-Tris(pH5.8)	20	5	10 ml
KCl	1000	1	40 µl
$MgSO_4.7H_2O$	250	0.8	128 µl
Boric acid	100	1.5	600 µl
$CaCl_2$	500	10	800 µl
PEG4000		15% w/v	6 g
Sucrose		5% w/v	2 g

Pollen collection & germination

Pollen grains were collected from freshly opened stage 13 flowers (Sanders et al., 1999). I use pollen from plants of ~ 5 weeks. Specific steps for each germination assay are:

1). Collect about 20 flowers and put into 1 ml germination medium.
2). Vortex for about 1.5 min to release pollen from dehisced anther.
3). Use a clean tweezer to remove the floating flower debris.
4). Centrifuge at a speed of 500g for 5 minutes at RT to pellet the pollen grains.
5). Remove 800 µl supernatant.
6). Vortex for 30 sec to release pollen grains from tube wall.
7). Transfer the remaining 200 µl medium containing the pollen grains into one well of the germination chamber (8 wells/chamber, Lab-Tek 155411).

8) Keep the germination chamber at 25 ºC in the dark (inside the Percival growth chamber) for 6 h before observation.

(I had determined the time course of tube growth. Growth rate is linear until about 8 h. So I chose 6 h).

Pollen tube measurement & observation

1.1. Take images of the germinated pollen in the same chamber with Nikon microscope (Nikon Eclipse E600). Images were saved automatically as Tif files. For quantification, I usually count at least 300 pollen grains (tubes). So the number of pictures per well I took depends on the pollen density. Usually I first select a field randomly, count the number of pollen, and then decide how many fields I use for image taking.
1.2. Use Scion Image (http://www.scioncorp.com/pages/scion_image_windows.htm) to analyze pollen tube length. Pollen tubes were randomly selected from the images and measured. Student's Test was used for statistics purpose.
1.3. Comments: I can get 20~70 % germination with this method.

References

Fan LM, Wang YF, Wang H, Wu WH (2001) In vitro Arabidopsis pollen germination and characterization of the inward potassium currents in Arabidopsis pollen grain protoplasts. J Exp Bot **52:** 1603-1614

Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant J **31:** 1-12

Sanders PM, Bui AQ, Weterings K, McIntire KN, Hsu YC, Lee PY, Truong MT, Beals TP, Goldberg RB (1999) Anther developmental defects in Arabidopsis thaliana male-sterile mutants. Sexual Plant Reproduction **11:** 297-322

Xiyan Li. Ph.D thesis. 2006.

1-b. DAPI staining of Pollen nuclei

Aim and Rationale.

A simple test of pollen development. Microsporogenesis involves two mitosis steps to yield 3 nuclei: one vegetative and 2 sperms. Mature pollen grains contain three nuclei which are visible by DAPI staining.

Reference (Modified from protocol on Preuss's webpage: http://preuss.bsd.uchicago.edu/index3.html?content=protocols/protocols.html)

1. Make working solution of DAPI (4'-6-Diamidino-2-phenylindole, Sigma, Cat. # D9564-10MG) (0.4 µg/ml) in ddH2O (can be kept in -4ºC for months).

2. Place pollen grains on a slide by dapping flowers (>100 pollen grains).

3. Add a few drops of DAPI solution. (no waiting time needed)

4. Cover slide and take pictures using microscope (Nikon Eclipse E600) observe under UV light.

Analyze images to get the percentage of tricellular, bicellular and unicellular pollen grains. Usually I use analyze at least 100 grains.

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1-c. Pollen Alexander staining (pollen viability assay)

Aim and Rationale

A test of pollen viability. Viable pollen grains are purple with a green outline and aborted pollen grains are green inside and out. The basis for this differential staining may be a pH difference in the cytoplasm between living and dead pollen

Alexander staining Solution

I mixed the following chemicals together.

Chemicals or solutions	Amount
95% Ethanol	10 ml
1% malachite green in 95% ethanol	1 ml
Distilled water	50 ml
Glycerol	25 ml
Phenol	5 g
Chloral hydrate	5 g
Acid fuschsin 1% in water	5 ml
Orange G 1% in water	0.5 ml
Glacial acidic acid	1 (-4) ml
Total volume- add water to	100 ml

(The acid is used for pH, and the amount to be used depends on thickness of the pollen cell wall. For Arabidopsis pollen, 1 ml is good enough)

Staining

1). Put a drop of the stain on the slide.
2). Mount pollen grains in the stain.
3). Cover with a cover slide.
4). Examine under microscope (Nikon Eclipse E600).

Reference

Alexander, M.P. 1969. Differential *staining* of aborted and nonaborted pollen. Stain Technology 44: 117-122

1-d. Hand-pollination (with limited pollen grains) and pollen fertility assay

Aim & rationale

Test if mutant pollen is less competitive in growth or is infertile. Each pistil contains 40-50 ovules, so pollinate with 35 grains to allow each pollen grain (including mutant) a chance to fertilize.

Materials

1). Pollen (♂): Arabidopsis *chx21* single mutant and mixture of *chx21* single and *chx21chx23* double mutant pollen.
2). Pistil (♀): imp-1 (Park et al., 2002). Wild-type plant except it is male sterile line (unless given Jasmonic acid). To prevent any self-pollination.

Procedure

1). Pollen grains from stage 13 flowers (Sanders et al., 1999) were put on a slide and counted. E.g. I used about 35 grains/per pistil.
2). Pollen grains were transferred to the stigma of the imp-1 pistil via the slide. (by bending the flower so stigma touches the slide). Pollinated flowers were marked with a color thread. For each type of pollen grains, 3~6 replicates were made. Important – Stage of the imp1 flowers can be recognized by the development of papillar cells. At stage 13, the papillar cells are fully developed and looked shining.
3). Put imp-1 plants back to the growth chamber.
4). Seven days after pollination, collect the 'marked' siliques from the imp-1 plants and take images under a stereoscopic zoom microscope (Nikon SMZ1000).
5). To observe seeds inside the pods, 70% ethanol was used to remove chlorophyll so the pods become more transparent (O/N).
6). To count seeds directly, siliques were opened with a dissection needle. Then seeds were collected and counted.

Reference

Park, J. H., R. Halitschke, et al. (2002). "A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis." Plant J **31**(1): 1-12.

Sanders, P. M., A. Q. Bui, et al. (1999). "Anther developmental defects in Arabidopsis thaliana male-sterile mutants." Sexual Plant Reproduction **11**(6): 297-322.

1-e. Hand-pollination (with limited pollen grains) and aniline blue staining to visualize *in vivo* pollen germination and tube growth

Aim & rationale

To test in vivo pollen germination and tube growth for the double mutant pollen. When same numbers of pollen grains were put on the imp-1 stigma, a chx21 and chx21chx23 mixture will give rise same number of tubes and same number of tubes reach the halfway of the pistil, if germination and tube growth is normal for the double mutant pollen. Shortcoming of this assay: single and double mutant cannot be distinguished. . Aniline blue stains callose**.**

Materials

Plant materials
1). Pollen (♂): Arabidopsis *chx21* single and *chx21chx23* double mutant pollen.
2). Pistil (♀): *imp-1*. (male sterile line)
Working solution
10% acetic acid in EtOH (fixative)
50 mM KPO_4 buffer: [4.17ml 1M K_2HPO_4 + 0.83 ml 1M KH_2PO_4 + 995 ml H_2O], pH 7.5
0.01% aniline blue in 50 mM KPO_4 buffer (dye)
Mounting medium: 50% glycerol made in the 50 mM KPO4 buffer

Procedure

1). Limited Hand-pollination was done the same as in the "Hand-pollination and pollen fertility assay" (A-1-4).
2). Six h after pollination, pollinated pistils from the imp-1 plants were collected with a tweezers.
3). Submerge pistil tissue in 1ml acetic acid and fix it for overnight.
4). Soften tissue by submerging it in 1 M NaOH overnight.
5). Wash three times with 50 mM K-PO_4 buffer at pH 7.5.
6). Stain with 200 µl 0.01% aniline blue for ~10 min.
7). Transfer the pistil to a slide, add mounting media and observe under UV.

Note: Try to avoid using UV before you take images. You can use Bright Field light to find your sample and get it focused. Even if you want to see the stained tubes before taking images, use partial strength of the UV light. Anyway, try not to bleach the fluorescence! (This is true for all fluorescence imaging, I think I got this from Steve Wolniak's Microscopy class.)

1-f. GUS staining to visualize chx21chx23 double mutant pollen tube growing in vivo

(Test of pollen tube growth & guidance in intact pistils)

Aim & rationale

As the single and double mutant pollen cannot be distinguished, Mark Johnson suggested we use SAIL mutants that have a T-DNA insertion carrying a LAT52 promoter-driven GUS. Only SAIL line we found was the *chx21-1s*. Crosses were made so a *chx21-1s+/- chx23-/-* line was obtained so only pollen carrying the 21/23 double would carry the GUS insertion and would stain blue. *chx23* single mutant would be colorless.

Materials

1). Pollen (♂):

Arabidopsis Wt_{GUS} (ecotype Col-0 background, From Dr. Palanivelu in Arizona);

SAIL mutant pollen in which T-DNA insertion carries a Lat52-driven GUS reporter (*chx21-1s*). Single 21 mutant or 21/23 double mutant

2). Pistil (♀): imp-1.

Solutions

Stocks:

10 % Triton X-100

0.5 M Na_2HPO_4 & 0.5 M NaH_2PO_4

100 mM Potassium Ferrocyanide ($K_4Fe(CN)_6$) (10.56 g/250 ml, store at 4 ºC in dark)

100 mM Potassium Ferricyanide ($K_3Fe(CN)_6$) (8.23 g/250 ml, store at 4 ºC in dark)

100 mM X-Gluc in dimethyl formamine (DMF) (0.104g/2ml)

Working solutions (50 ml)

0.2 % Triton X-100

50 mM $NaHPO_4$ buffer (68.4 part Na_2HPO_4 + 31.6 part NaH_2PO_4) (pH 7.2)

2 mM Potassium Ferrocyanide ($K_4Fe(CN)_6$)

2 mM Potassium Ferricyanide ($K_3Fe(CN)_6$)

H_2O

1 ml X-Gluc stock

Procedure

1). I hand-pollinated the *imp-1* pistil [flower] with Wt_{GUS} and mutant pollen. Pollen grains from one flower were pollinated to one *imp1-1* pistil.

2). Certain times after pollination (6, 9, 24 h), pollinated imp-1 flowers were excised with a tweezers.

3). Under microscope (Nikon SMZ1000), I dissect and remove ovary wall materials.

4). Place the intact ovules in 80% acetone O/N for fixation. [1 ml 80% acetone in 1.5 ml eppendorf tube

5). Put the fixed ovules in 1 ml X-Gluc. Incubate at 37 ºC for 1 h.

7). Observe under microscopes (Nikon SMZ1000 and Nikon Eclipse E600).

Analysis and quantitation:

I normally do 5~6 flowers for each type of pollen. I repeated this expt. more than 3 times to confirm the results.

1-g. Semi *in vivo* pollen tube guidance assay

Aim and rationale

Hypothesis: Ovule produces chemical cues that attract the growth of pollen tubes in vivo. Pollen tubes are primed to respond to ovule cues only after tubes grow into the style. Therefore this assay measures whether pollen tubes (wild-type & mutants) will respond to ovule cues after initial germination and tube growth in the pistil.

Materials

Plant materials

1). Pollen (♂): Arabidopsis Wt_{GUS} (ecotype Col-0) and mutant pollen.

2). Pistil (♀): imp-1.

GUS staining solution

The same as that in the **A-1-6** protocol.

Pollen tube growth plate

18% Sucrose

0.01% Boric acid (1.6 mM)

1mM $CaCl_2$

1mM $Ca(NO_3)_2$

1mM $MgSo_4$

0.5% agar

Note: a). adjust pH to 7.0 with 1 M KOH before adding agar;
b). microwave the medium to dissolve agar; [No autoclave needed]
c). pour completely dissolved medium into petri dishes (Falcon Easy Grip petri dishes, 35 x 10 mm style);
d) keep plates at 4 ºC.

Procedure (Modified from (Palanivelu and Preuss, 2006))

1). Hand-pollinate one pistil with pollen grains from about ¼ anther.

2). Right after pollination, cut pistil at the shoulder of the ovary and put on the medium plate (lay on its side).

3). Isolate ovules: Dissect pistil (stage 13 flowers) and isolate intact ovule with the stalk. (do this under stereo microscope)

4). Place 4 ovules around each cut pistil on the plate within 150 um distance.

5). Put the covered petri dishes into a box [25cm*25cm*15cm] with wet paper towel and cover. incubate @ 25 ºC for about 4 h, till tubes reach ovules.

6). Add GUS staining solution (~10 µl) to a dent I cut on the plate next to the ovules . GUS staining solution diffuses into the agar plate and stains the tubes. This prevents flooding which cause ovules to drift away.

7). Keep plate @ 37 ºC for about 0.5 h.

8). Observe under microscopes (Nikon SMZ1000 and Nikon Eclipse E600).

Microscopy: I used the scope on the 3rd floor, the reason I used that one is because it is an inverted scope, which is good when you observe cells in petri dishes. I used both 10X and 20X magnifications.

Note: Use very fresh flowers/plants and fresh plates (best freshly made).

Reference

1) Tube growth medium: Dr. Ravi Palanivelu's webpage: http://www.ag.arizona.edu/research/ravilab/lab%20protocols%20page.html.

2) **Palanivelu R, Preuss D** (2006) Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro. Bmc Plant Biology **6:** -

3). Lu visited Ravi's lab in Oct. 2008, and learned to conduct assay.
Special Tips from Ravi :
1). Put isolated ovules around the cut pistil within about 150 µm;
2) try to use relatively same amount of pollen grains, like I used pollen grains from ~1/4 anther for each imp-1 pistil.

2. RNA expression

2-a. RNA extraction from Pollen

Aim

I used this procedure to extract mRNA in order to determine CHX expression in pollen, and in seedlings by RT-PCR.

Beforehand preparation

Drill tips were rinsed, wiped, chloroform-rinsed (in the fume-hood) and wiped again and then autoclaved.

Pollen collection (based on Honys and Twell (2003)

1). Collect flowers and put into a large flask. (I used 1 tray of plants, 12 pots with about 10 plants/pot)
2). Add 300 ml ice-cold 0.3 M mannitol.
3). Shake the flask vigorously shaken for 2 min.
4). Filter the pollen suspension through 100 µm nylon mesh.
5). Collect pollen by repeated centrifugation steps (50-mL Falcon tubes, 450 g, 5 min, 4°C).
6). Store pollen pellet in 1.5 ml centrifuge tubes at -80 ºC.

Homogenization

1). Take out pollen from -80 ºC and put into liquid N_2.
2). Homogenize pollen with drill.
3). Add TRIzol reagent (1 ml reagent/ 50-100 mg tissue, the sample volume should not exceed 10% of the volume of TRIzol used for homogenization).

Phase separation

1). Incubate the homogenized samples for 5 minutes at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes.
2). Add 0.2 ml of chloroform per 1 ml of TRIzol reagent. Cap sample tubes securely.
3). Shake tubes vigorously by hand for 15 seconds and incubate them at RT for 2 to 3 minutes.
4). Centrifuge the samples at no more than 12,000 x g for 15 minutes at 4 °C. (Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol reagent used for homogenization).
6). Transfer the aqueous phase to a fresh tube.

RNA precipitation

1). Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol (Use 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent used for the initial homogenization).
2). Incubate samples at RT for 10 minutes.

3). Centrifuge at no more than 12,000 x g for 10 minutes at 4 °C.
4). Discard the supernatant.

RNA wash

1). Wash the RNA pellet with 75% ethanol (use at least 1 ml of 75% Ethanol per 1 ml of TRIzol reagent used for the initial homogenization).
2). Mix the sample by vortexing.
3). Centrifuge at no more than 7,500 x g for 5 mintues at 4 °C.
4). Discard the supernatant.

Dissolving RNA

1). Briefly dry the RNA pellet
2). Dissolve RNA in (~ 50 ul Lu guessed) RNase-free water by passing the solution in a few times through a pipette tip.
3). Incubate for at 55 to 60 °C 10 minutes.
4). Tap the tube several times.
5). Store the RNA in -80 °C for further use.

Recovery: I got about 50 ul and ? ug RNA.

Reference

Honys, D. and D. Twell (2003). "Comparative analysis of the Arabidopsis pollen transcriptome." Plant Physiol **132**(2): 640-52.

2-b. RT-PCR

Aim: Determine the expression of CHX genes in pollen and in seedling.

First strand cDNA synthesis:

(Invitrogen SuperScript first-strand synthesis Kit. Cat. No. 11904-018)

1. Mix and briefly centrifuge each component before use.
2. Prepare RNA/primer mixtures in sterile 0.2 ml tubes as follows:

Total RNA [template]	5 µl
10 mM dNTP mix	1 µl
Oligo$(dT)_{12\text{-}18}$ (0.5 µg/ µl)	1 µl
DEPC-treated water	3 µl

3. Incubate each sample at 65 °C for 5 min, then place on ice for at least 1 min.
4. Prepare the following reaction mixture, adding each component in the indicated order.

10X RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 M DTT	2 µl
RNaseOUT Recombination RNase Inhibitor	1 µl

5. Add 9 µl of reaction mixture to each RNA/primer mixture, mix gently, and then briefly centrifuge.
6. Incubate at 42 °C for 2 min.
7. Add 1 µl (50 units) of SuperScript II RT to each tube except the no RT control, mix, and incubate at 42 °C for 50 min.
8. Terminate the reaction at 70 °C for 15 min. chill on ice.
9. Collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 min at 37 °C before proceeding to Amplification of the Target DNA.

Amplification of the Target DNA

1. DNA polymerase: Tag DNA polymerase
2. Loading control: Actin11
3. PCR cycles: 30

Comments:

I used ~0.3 ul pollen RNA for each PCR, and ~ 2 ul seedling RNA for PCR.

3 Molecular cloning

3-a Revised CHX23 cDNA cloning (pECHX23_110807)

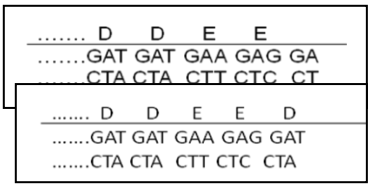
Aim: To revise the incorrect cDNA sequence

Problem: The lab already had the CHX23 cDNA before I started my project, but I found the C-tail had a deletion (22 b before the stop codon, so the seq was not in frame, so I added one more base and make it in frame. Now, compared to the predicted full length protein, 7 aa was missed.

C-terminal sequence of the old CHX23 cDNA: ….

The correct sequence should be as bottom panel:

So, a T will be added to the C terminal of the 1st CHX23 cDNA.



PCR amplification:

Enzyme for the PCR amplification: Plantinum Pfx DNA Polymerase

Template: the old CHX23 cDNA

Primers: CHX23Cf and CHX23Cr (see the primer table)

PCR cycle: 30

Temperatures: denaturing, 94 ºC, annealing, 53 ºC, elongating, 68 ºC.

Entry vector cloning:

Enzyme: Invitrogen Gateway BP Clonase™ II Enzyme Mix (cat. #11789-020)

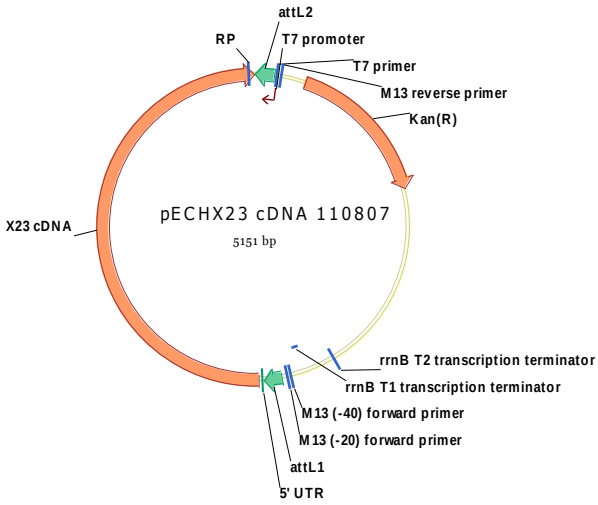
Donor vector: Invitrogen pDONR221

Note: after purification, the new entry clone was confirmed in two ways sequentially:

1). Enzyme digest;

2). Sequencing (see the primer table for the sequencing primers).

pECHX23 vector map



3-b. Constructing protoplast expression vectors
(pD2GWF/R-CHX23 vector)

Aim: for transient expression in isolated Arabidopsis leaf mesophyll protoplast cells

LR reaction:

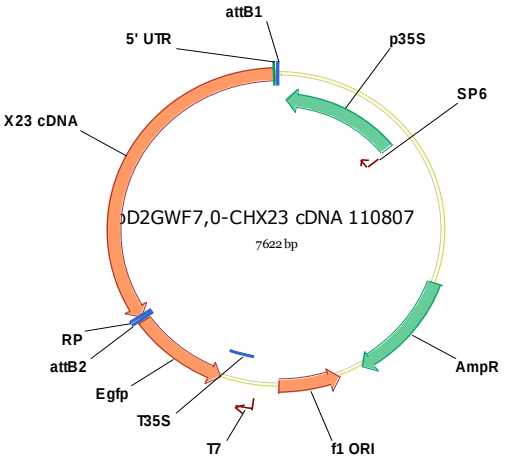
1. Entry clone: pECHX23_110807
2. Destination vector: p2GWF7/p2GWR7
3. Enzyme: Invitrogen Gateway LR Clonase™ II Enzyme Mix (cat. #11791-020)

Expression clone check:

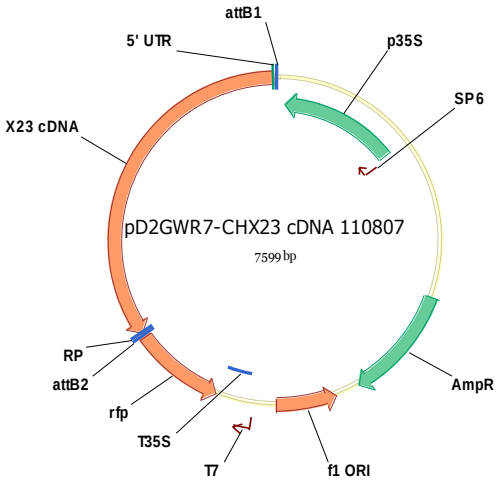
Enzyme check: use BamH I and then Sac I, checked twice.

Vector maps:

pD2GWF7-CHX23



pD2GWR7-CHX23



3-c. CHX23 yeast vector cloning
(pYESDR196/pGWFDR196-CHX23)

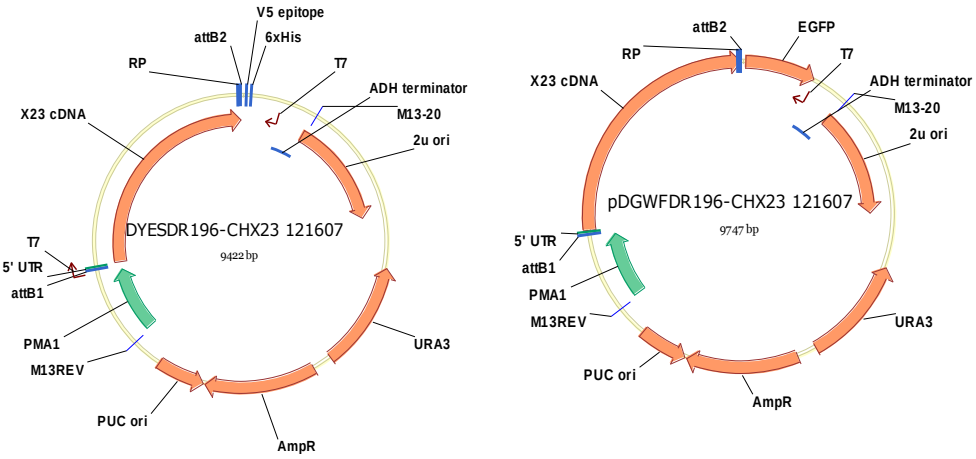
LR reaction:

1. Entry clone: pECHX23_110807
2. Destination vector: pYESDR196/pGWFDR196
3. Enzyme: Invitrogen Gateway LR Clonase^{TM} II Enzyme Mix (cat. #11791-020)

Expression clone check:

Enzyme check: use BamH I and then Sac I, checked twice.

pDYESDR196/pGWFDR196-CHX23 map:



3-d. CHX23 E.Coli vector cloning

(By Lalu Zulkifli in Nobuyuki Uozumi lab)

1) The primers Lalu used for PCR-amplification of CHX23 cDNA were:
   AtCHX23-BglII-SE: GAGAGATCTATGTCTTCCGGAGCCCC
   AtCHX23-XhoI-AN:ATTCTCCAG TTACTCATGAATTCCATATTGATG
2) The clone sequence Lalu got was terminated at I_{778}, with 89 aa missed compared to the predicted full length.
3) The primers I am using to amplify the CHX23 cDNA are:
   AtCHX23-Bgl II-SE**: GA AGA TCT TC** ATG TCT TCC GGA GCC CCC CTT AAT GT
   ATCHX23-XhoI-AN: **CCG CTC GAG CGG** TTA ATC CTC TTC ATC ATC TTC ATC TTC GTC TTC
4) The clone sequence I will get is terminated at D_{860}, with 7 aa missed compared to the predicted full length.

3-e. Lat52 vector cloning (pLatGWF7, pLatGWR7, pB7FWGLat)

(Assisted by Guoying Wang)

Aim: for Gateway vector expression in pollen

PCR amplification of Lat52 promoter sequence

Template: Lat52 plasmid from Alice Cheung lab (Lat52-GFP-NtADF1)
Enzyme: Platinum Pfx DNA polymerase
Primers: Lat52 Pf and Lat52 Pr (see primer table)
Cycles: 30

PCR product purification

Purification kit: illustra™ GFX PCR DNA and Gel Band Purification Kit (GE healthcare 28903471)

Digestion

Enzyme: Sac I and Spec I
Templates: 1). Vectors: p2GWF7, p2GWR7 and pB7FWG2
2). Purified PCR product
Conditions: 37 ºC for 2.5 h

Ligation

Use the Quick T4 DNA Ligase kit (NEB, cat# M2200S) and follow the instructions

Transformation

Cell strain:

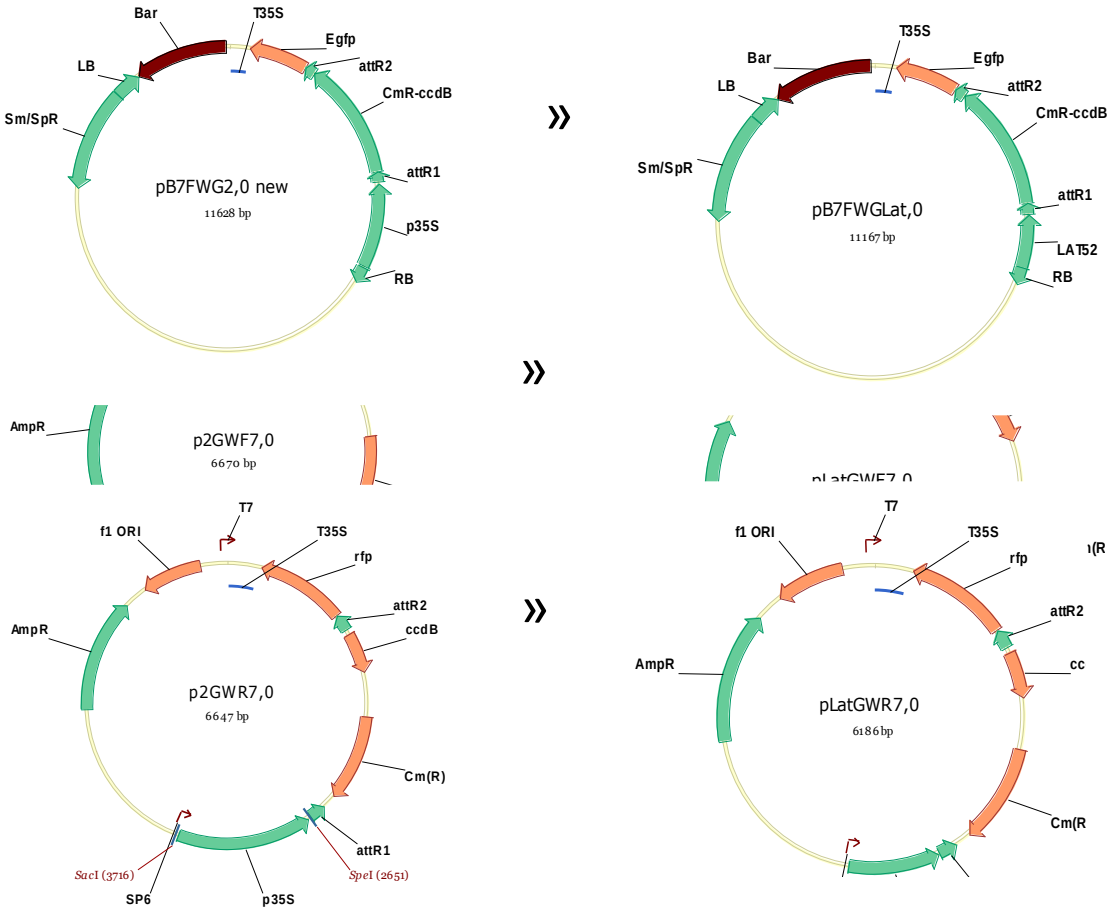
Plasmid collection

Use the Qiagen kit (QIAprep Spin Mini prep Kit, Cat. # 27106).

Vector checking

1). Enzyme digestion: Sac I and Spe I
2). Sequencing: use primers Lat52 Pf and Lat52 Pr (see primer table)

Vector maps: pLatGWF7, pLatGWR7, pB7FWGLat



3-f. Lat52 driven CHX23-GFP/RFP vector cloning

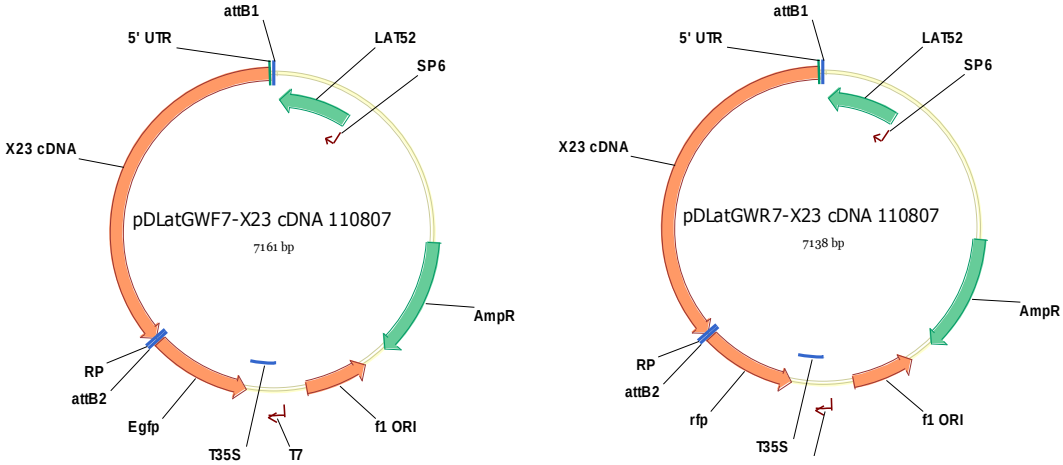
LR reaction (follow the manufacturer's instructions):

1. Entry clone: pECHX23 (the new clone that has the C-tail in frame)
2. Destination vector: pLatGWF7/pLatGWR7/ pB7FWGLat
3. Enzyme: Invitrogen Gateway LR Clonase™ II Enzyme Mix (cat. #11791-020)

Expression clone check:

1. Enzyme check: use Sca I and Spec I.
2. Sequencing: with Lat52 Pf primer (see the primer table)

Vector maps:



3-g. Genomic CHX23 entry cloning (pECHX23pro+cod-stp)

PCR amplifying the genomic region

Enzyme: Plantinum Pfx DNA Polymerase

Template: BAC clone (F_3F_{20})

Primers: CHX23GCf and CHX23GCr (see the primer table)

PCR Cycle: 25

Gene purification

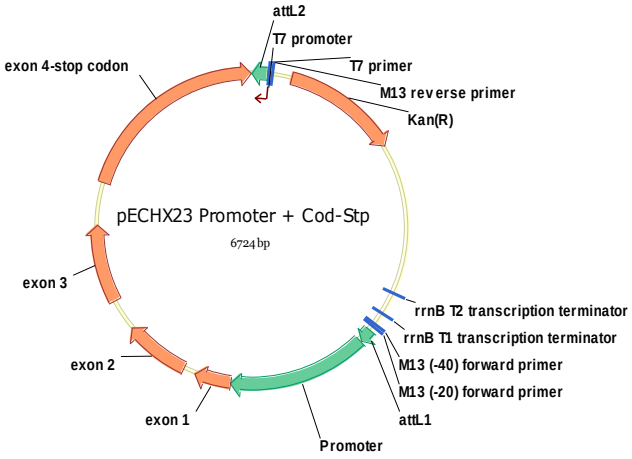
Purification kit: illustra^{TM} GFX PCR DNA and Gel Band Purification Kit (GE healthcare 28903471).

Gateway Entry vector cloning (BP reaction)

Enzyme: Invitrogen Gateway BP Clonase^{TM} II Enzyme Mix (cat. #11789-020)

Donor vector: Invitrogen pDONR221

Note: after purification, the new entry clone was confirmed in two ways sequentially: 1). Enzyme digest; 2). Sequencing (see the primer table for the sequencing primers).



3-h. Genomic CHX23 expression cloning (pDMDC107-CHX23)

Materials:

Enzyme: Invitrogen Gateway LR Clonase™ II Enzyme Mix (cat. #11791-020)

Entry vector: pECHX23pro+cod-stp

Destination vector: pMDC107 (Curtis and Grossniklaus 2003)

Procedure (as the entry clone and the destination vector has the same bacterial antibiotics gene, this part will give more details of how to resolve this problem):

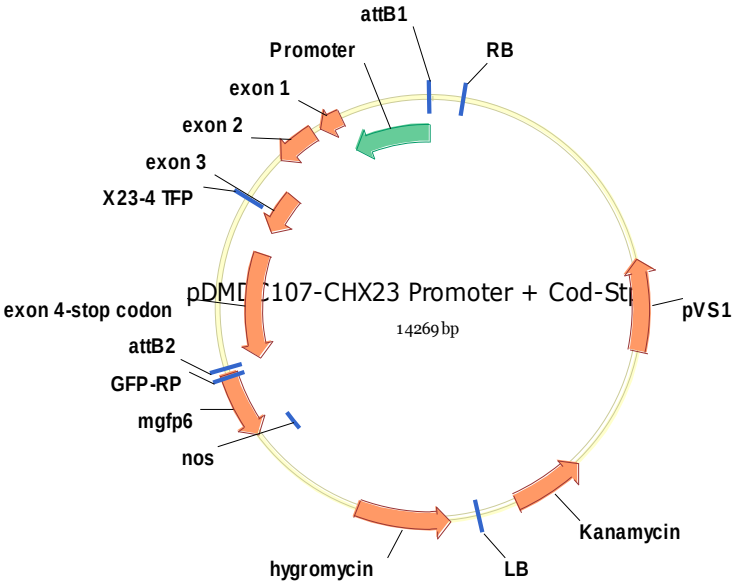
1. LR reaction (follow the manufacture instructions).
2. Add enzyme mlul to the LR solution and keep reaction at 37 ºC for 4 h (*the entry clone carries a single mlul site outside of the genomic CHX23 region. There is also a single mlul site on the pMDC107 destination vector, but within the region of the recombination sites. So after LR reaction, the mlul enzyme only cut left entry clone and pMDC107 vector, but not LR product CHX23 expression clone. Because in the expression clone, the mlul site has been change to the CHX23 sequence after recommbination).
3. Terminate the mlul reaction with protease K (at 37 ºC for 10 min).
4. Use the solution to transform E.Coli competent cells (One Shot OmniMAX 2 T1 Phage-Resistant Cells, invitrogen cat. #C8540-03).
5. Inoculate the transformant cells directly into LB medium + Kanamycin without plating.
6. Grow cells at 37 ºC and then collect plasmids using the Qiagen kit (QIAprep Spin Mini prep Kit, Cat. # 27106).
7. Run a gel to separate the plasmids (entry clone vs. expression clone).
8. Cut the gel containing the expression clone and purify the plasmid.
9. Dilute the plasmid 50 times.
10. 2nd time E.Coli transformation and plating on LB agar medium with Kanamycin.
11. Pick up colonies and inoculation.
12. 2nd time plasmid collection.

New vector was double checked: 1) Enzyme digest expression clone with Xho I, that produce three digestion products in the size of 1094, 2067 and 111086 bp. Use entry clone as a control when run the gel; 2). Sequencing with the M13F(-21) primer to make sure the border sequence was not affected after the recombination reaction.

Note: the major problem for this cloning is that the donor vector and destination vector has the same bacterial antibiotics gene (Kanamycin in this case). The key points to resolve this kind of problem are: 1). Find an enzyme that cut the donor vector outside of the recombination site, but cut destination vector within the recombination site, or no cutting site on the destination vector. Neither can this enzyme cut the gene of interest; 2). There should be difference in the size between donor vector and destination vector, so you can separate them by running a gel (even you use the enzyme, there are always some entry vectors left). Usually Gateway binary vectors are huge and donor vectors are small in size, so there should not be problem to separate them.

Curtis, M. D. and U. Grossniklaus (2003). "A gateway cloning vector set for high-throughput functional analysis of genes in planta." Plant Physiol **133**(2): 462-9.

pDMDC107-CHX23 vector map:



4. Protein membrane localization

4-a Transient CHX23 gene expression in isolated Arabidopsis leaf mesophyll protoplast

Aim: Isolate protoplasts to determine membrane localization of CHX23-tagged with fluorescent protein (GFP or RFP)

Protoplast isolation

Plant materials:

~ 3 week-old wild-type Arabidopsis thaliana (ecotype Col-0) grown under short light conditions (12 h light, 50-150 µE).

Procedure:

1) Make 5 ml enzyme solution (enough for 4~5 transfections) and pour into petri dishes (Falcon Easy Grip petri dishes, 35 x 10 mm style).
2) Cut 0.5~1 mm leaf strips with fresh razor blade without wounding. Transfer the strips immediately to the enzyme solution. Cut about 20 leaves.
3) Apply vacuum for 30 minutes.
4) Wrap the plate with foil and digest the leaf tissue for 3 h at RT w/o shaking.
5) Filter the enzyme solution containing the protoplasts with a nylon mesh (75 µm) into a round-bottom tube (12 × 75 mm, Fisher cat# 14-956-3C).
6) Rinse the plate with W5 solution and filter and combine the filter-through.
7) Spin at 100 g for 1.5 minutes to pellet the protoplast (speed 3 with the IEC clinical centrifuge).
8) Discard the supernatant and resuspend protoplasts with same volume of W5 solution.
9) Spin at speed 3 for 1.5 min, discard the supernatant and resuspend with 1 ml W5 solution.
10) Count cells using a chamber counter. Get a final cell density of about 2.5×10^5/ml.
11) Keep the protoplast on ice for 30 min.
12) Pellet the protoplasts (speed3 for 2 min) and resuspend in same volume of MMg solution.

Note: always use fresh, young and healthy leaves that looked round, pale-green color and flash. Never use leaves that looked dark-green, stressed and old.

Protoplast transformation

1) Coat the round-bottom tube with 5% fetal bovine serum (FBS) and remove the leftover.
2) Add 25 µl DNA (~ 10 µg plasmid of about 5 k in size). For cotransfection, use half of the amount for each DNA and the total keep the same.
3) Add 150 µl protoplast and mix well by gently rocking around the tube.
4) Add 175 µl PEG/Ca solution (equal to the volume of 25 µl DNA + 150 µl protoplast) and mix well by gently rocking and rolling the tube. Incubate at RT for about 20 min.
5) Dilute with 3 ml W5 solution and mix well gently.
6) Spin at speed 3 for 2 min and remove the supernatant.
7) Resuspend protoplast in 200 µl WI solution.
8) Wrap tube with Al foil and keep at RT for O/N.
9) Observe under microscope.

Note:
1). It is the purity of the DNA that make difference! (I recommend using the QIAGEN HiSpeed Plasmid Midi Kit, Cat# 12643, for the DNA plasmid preparation);
2). During the protoplast transformation, try to be as gently as possible even when you are doing the mixing.

Confocal microscopy (Zeiss LSM510)

Filter setting:

Fluorophore	Excitation (nm)	Emission (mm)
GFP	488	BP 505-530
RFP	543	BP560-615
Chlorophyll	488	LP 650

Note:

1. Try to use low dose of laser before you taking images, otherwise the fluorescence goes very fast.

2. Try to minimize the pin hole, even at the cost of higher laser strength (for me, I always put more weight on pin hole. Why? Just think about the theory of confocal imaging!).

Solutions

1. Enzyme solution (5 ml)

Stock	Volume	Working concentration
1 M Mannitol	2 ml	0.4 M
1 M KCl	0.1 ml	20 mM
0.5 M MES, pH 5.7	0.2 ml	20 mM
Cellulase R10	50-75 mg	1-1.5 %
Macerozyme R10	10-20 mg	0.2-0.4 %
Heat the enzyme solution at 55 ºC for 10 min (to inactivate protease and enhance enzyme solubility) and cool down to RT before adding the following		
1 M CaCl2	0.05 ml	10 mM
β-mercaptoethanol	2 µl	5 mM
10 % FBS (Sigma F6178)	0.05 ml	0.1%

2. PEG solution (40%, w/v) 10 ml

PEG4000 (Fluka, # 81240)	4 g	40%, w/v
1 M Mannitol	2 ml	200 mM
1 M CaCl2	1 ml	100 mM
H2O	3.5 ml	

3. W5 solution (50 ml)

1 M NaCl	7.7 ml	154 mM
1 M CaCl2	6.25 ml	125 mM
1 M KCl	0.25 ml	5 mM

0.5 M MES, pH 5.7	0.2 ml	2 mM

4. MMg solution (5 ml)

Stock	Volume	Working concentration
1 M Mannitol	2 ml	0.4 M
1 M $MgCl_2$	75 µl	15 mM
0.5 MES, pH 5.7	40 µl	4 mM

5. Washing & Incubation solution (WI) (10 ml)

1 M Mannitol	5 ml	0.5 M
1 M KCl	0.2 ml	20 mM
0.5 M MES, pH 5.7	80 µl	4 mM

This protocol is based on the one from Jen Sheen's lab:
http://genetics.mgh.harvard.edu/sheenweb/

A movie of how to isolate protoplast and then transformation is also available on her lab website and is highly recommended to watch!

4-b. Transient gene expression in tobacco pollen after bombardment

(From: Alice Cheung lab, U. Mass. Jan 2009. YX Lu visited lab and did expt there.)

Aim: Localize CHX23 protein fused to fluorescent proteins after expression in tobacco pollen

DNA precipitation (concentrate DNA into 1~2.5 µg/µl, DNA was collected using the QIAGEN HiSpeed Plasmid Midi Kit):

1) Mix 1 Vol DNA with 1/10 Vol 3 M NaAc and 2~2.5 Vol Ethanol.
2) Put in -20 ºC for O/N.
3) Centrifuge @ 14.8 K RPM for 10 minutes. Discard the supernatant.
4) Add 600 µl 75% ethanol, votex, spin again @ 14.8 K rpm for 5 min. Discard the supernatant.
5) Vacuum dry the DNA pullet for ~15 min.
6) Add TE buffer or H_2O to make the final DNA concentration as ≥2.5 µg/µl.
7) OD assay: 0.35~0.5.
8) Calculate DNA conc. From OD value: OD reading *100*50 µg/ml. times 100 is because 100 times dilution when test OD.
9) Accordingly, Lat52-x23-gfp conc. is 1.765 µg/µl, Lat52-x23-rfp is 2.13 µg/µl.

Particle preparation

1). Add 1 ml freshly made 70% ETOH to 60 mg of tungsten particles.

2). Vortex vigorously for at least 30 mins, longer is better.

3). Spin 2 sec and remove the ETOH.

4). Add 1 ml sterile dH_2O, spin 2 sec, remove supernatant; repeat twice more.

5). Resuspend in 1 ml of sterile 50% glycerol

Note (from Alice Cheung lab): The above particles are supposed to be stable for up to two weeks at RT, but keeping at 4 ºC or -20 ºC could only be better. We have used particles kept at -20 ºC for at least two weeks.

Particle coating: (everything should be on ice)

1). Vortex the activated particles at maximum speed for at least 30 min to resuspend them well.
2). Determine DNA amount: for the 1st experiment, the amount of the DNA is as following:
Lat52-x23-gfp: 2.5 µg, so 1.41 µl;
Lat52-x23-rfp: 2.5 µg (1.17 µl) + Lat52-GFP 0.5 µg (0.5 µl).
(Note: Lat52-GFP was added with Lat52-x23-rfp to make the RFP signal easier detected).

3). Vortex the activated particles vigorously to resuspend them well, then quickly pipet 25 µl to the DNA tube, vortex immediately to mix well.
4). Add 25 µl 2.5 M $CaCl_2$, vortex immediately to mix well.
5). Add 10 µl 0.1 M spermidine, vortex immediately to mix well.
6). Vortex the tube vigorously for 20 min (periodically put tube back to ice).
7). Spin ~15 S, remove the supernatant.
8). Add 200 µl 70% Ethanol, vortex ~ 1 min, twice.
9). Spin ~ 15 S, remove the supernatant.
10). Add 200 µl 100% ethanol, vortex ~1 min, twice.
11). Spin ~ 15 S, remove the supernatant.
12). Resuspend particles in 20 µl 100% ethanol. Ready for macrocarrier loading.

Pollen petri dish & germination slide preparation

1). Prepare 3.5 mm petri dishes with 1 ml of pollen germination medium (Basal + 0.7 % Agarose).
2). Make germination slide by adding 0.5 ml melted medium on a slide, put slides inside wet box.
3). Prepare 7 mg pollen (in 100 µl germination medium) and pour on the petri dish, spread out the pollen on the dish surface.
4). Air-dry the dishes. (note: cannot be too wet, cannot be too dry either. When the pollen looked like transparent gel state).

Bombardment (helium-driven PDS-1000/He biolistic system, Bio-Rad, Hercules, CA)

1). Switch on hlium till get the pressure reading as ~14 K PSI.
2). Turn on vacuum pump (far left-hand switch).
3). Load rupture disc into holder, put holder into position and tighten a little bit.
4). Load stopping screen 1st, and then macrocarrier, and put the whole assembly into position.
5). Put pollen petri dish into position.
6). Close the chamber door.
7). Push the middle button to upward position to start vacuum.
8). After the gauge reading reaches ~24 PSI, flip the button quickly to the downward position to hold the vacuum.
9). Push the far right-hand switch and hold it, this lets the helium in, once you hear the rupture sound, release the button.
10). One bombardment is complete.
11). Use liquid pollen germination medium to wash and transfer pollen from the petri dish to the germination slides, usually 400 µl for 4 slides.
12). Transfer the slide holding pollen into wet box for germination.
13). Put the wet box into 30 ºC, 4 h later do the microscopy work.

After-done work

1). Push the middle button to center position to vent the chamber, the vacuum gauge will return to 0.

2). Open the chamber, discard the stopping screen/macrocarrier and rupture disc in order.
3). Put everything inside the chamber.
4). Close the door.
5). Start to vacuum the chamber again till reading is high.
6). Stop the helim supply by witch off the tank outlet.
7). Push the far righ-hand button to release helium residue into the chamber, hold for 1 min, release it, push it again and hold for a few seconds, till gauge above the helium tank is read 0.
8). Vent the chamber.
9). Turn off the vacuum pump.
10). Sign off the log book.

Pollen germination medium

0.01% H_3BO_3, 0.01% HNO_3, 0.02% $MgSO_4$, 0.07% $CaCl_2$, 15% PEG-3350, 2% Suc, and 20 mM Mes, pH 6. For solid medium plate, remove PEG and change Suc from 2% to 8%.

4-c. CHX23 gene expression in Stably-transformed Arabidopsis

Aim: I determined CHX23-GFP localization in pollen from plant that was stably transformed with a native promoter-driven CHX23-GFP construct.

Materials

Binary vector: pMDC107-CHX23
Bacteria: Agrobacterium tumefaciens strain GV3101.
Plant: Wild-type Arabidopsis thaliana (ecotype Col-0).

Transformation:

Bacteria: electroporation
Plant: floral dip (Clough and Bent, 1998)

Transformant screening.

Bacteria: gentamicin (50 µg/ml) and Kanamycin (50 µg/ml).
Plant: hygromycin (30 µg/ml)

Transformant pollen collection & germination

See *In vitro* pollen germination **(A-1-1).**

Confocal pollen imaging

See Transient gene expression in isolated Arabidopsis leaf mesophyll protoplast **(A-4-1).**

Reference

Clough, S. J. and A. F. Bent (1998). "Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana." Plant J **16**(6): 735-43.

5. CHX23 transporter assays

5-a Assays in yeast cells

Aim: Test CHX23 transporter activity in action/proton exchanger yeast mutant cells.

Making yeast competent cells (Gietz and Schiestl, 2007)

1). Make YPAD plate & liquid medium:
1% Bacto-Yeast Extract – 10 g/L
2% Bacto-Peptone – 20 g/L
2% Glucose (Dextrose) – 20 g/L
If making YPD plates, add 20 g Bacto-agar.
Fill up to 1 liter with deionized HOH.
NO pH adjustments

Note: autoclave agar & glucose separately, to avoid caramelization.

2). Get the yeast strains and streak on the YPAD plates. Let cells grow 2 d before inoculation.

3). Inoculate one colony into 25 ml YPAD liquid medium and grow cells O/N @ 30 C with shaking speed around 200 rpm.

4). 2nd inoculation. Put the 25 ml cell culture into 75 ml YPAD medium. Grow cells @ 30 ºC for 4 h.

5). Harvest cells by centrifugation @ 3000 g for 5 min, wash cells with 0.5 volumes of sterile H_2O, pullet, and re-suspend in 0.01 volumes of sterile H_2O, transfer to a suitable centrifuge tube and pellet @ 3000 g for 5 min @ 20 ºC.

6). Re-suspend cell pellet in 0.01 volumes of filter sterile frozen competent cell solution (5 % v/v glycerol, 10% v/v DMSO).

7). Dispense 50 µl cells into 1.5 ml appendorf tubes.

8). Place the tubes into box with Styrofoam or cardboard. (Slowly freezing is essential for good survival rates).

9). Put the box into -80 freezer. (Cells can be kept at this condition for up to one year).

Yeast transformation

1). Thaw cells @ 37 ºC water bath for 15-30 S.

2). Centrifuge @ 13,000 g for 2 min to remove the supernatant.

3). Make up the transformation solution for the planned number of transformation + one extra (negative control). Add the mixture to the cell pellet, votex to re-suspend the cells.

Components	Volume (µl)
PEG 3350 (50 % (w/v))	260
LiAc 1 M	36
Salmon sperm DNA (10mg/ml)	10
Plasmid	3
Sterile H2O	51
Total	360

4). Incubate in a 42 ºC water bath for 40 min.
5). Centrifuge @ 13,000 g for 30 s and remove the supernatant.
6). Pipette 1 ml of sterile H_2O into the transformation tube to re-suspend the pellet.
7). Plate 200 ul of the cell suspension onto the YNB+MA plate

YNB+ MA plate (100 ml):
YNB: 0.67 g
MES (20 mM): 0.39 g
ADE: 0.01 g
(Adjust to pH 5.7 with Arg)
Agar: 2 g
Glucose: 2 g

8). Incubate plates @ 30 ºC for 3~4 d.

Yeast cell growth assay (Drop test)

1). Pick a tranformant colony and inoculate to the liquid YNB medium (5ml) with ampicilin selection marker and culture @ 30 ºC for O/N.
2). Use 1 ml cell culture and dilute with YNB w/o sucrose 6 times, culture @ 30 ºC for O/N.
3). Measure OD with spectrophotometer.
4). Make 10-time series dilutions.
5). Spot 5 µl to the plate.
6). Grow cells @ 30 ºC for 2 or 3 d, then observe.

Gietz, R. D. and R. H. Schiestl (2007). "Frozen competent yeast cells that can be transformed with high efficiency using the LiAc/SS carrier DNA/PEG method." Nat Protoc **2**(1): 1-4.

5-b assays in bacteria: Growth and K^+ content

(Experiments conducted by Lalu Zulkifli From Nobuyuki Uozumi's lab, 2009, Japan)

CHX23 bacterial vector cloning:

1) PCR amplifying CHX23 cDNA with Primers AtCHX23-BglII-SE & AtCHX23-XhoI-AN using pECHX23 entry clone as template.
   AtCHX23-BglII-SE: GAGAGATCTATGTCTTCCGGAGCCCC
   AtCHX23-XhoI-AN: ATTCTCCAG TTACTCATGAATTCCATATTGATG
2) Purify PCR product from agarose gel.
3) Digest purified PCR product with BglII/XhoI.
4) Ligate the digested product into pPAB404 vector.
5) Transform E.Coli XL10 Gold with the new vector
6) Grow cells on LB-amphicillin plate @ 37 ºC for O/N.
7) Inoculate and grow cells O/N.
8) Isolate plasmid.
9) Check the new clone with colony PCR and sequencing.

Cell growth & K+ content assay

1) Transform the new bacterial vector into E.Coli strain LB2003 that lack three K+ uptake transporters (Stumpe and Bakker, 1997).
2) Cell growth assay: on medium containing varied [10-30 mM] concentrations of K and at different pH's. Ability to grow on low K-containing medium means cells are able to take up K. [based on Matsuda et al (2004)].
3) K content assay: see methods in YX Lu thesis or manuscript. Methods based on Matsuda et al (2004).
   Principle: Measure K content in cells at varied pH's and as function of time.
   - Incubate K^+-depleted cells harboring CHX23 or the empty vector with 20 mM KCl in buffers at pH 4.3, 5.6 or pH 7.5 containing 10 mM glucose.
   - Aliquot at the times indicated in Fig. x. One mL of the cell suspension was taken at the indicated time and transferred into a tube containing 150 µl of silicon oil, and subsequently centrifuged at 12,000 rpm for 1 min.
   - Determine the K+ content by flame photometry.

Stumpe, S. and E. P. Bakker (1997). "Requirement of a large K+-uptake capacity and of extracytoplasmic protease activity for protamine resistance of Escherichia coli." Arch Microbiol **167**(2/3): 126-36.

Matsuda, N., H. Kobayashi, et al. (2004). "Na+-dependent K+ uptake Ktr system from the cyanobacterium Synechocystis sp. PCC 6803 and its role in the early phases of cell adaptation to hyperosmotic shock." J Biol Chem 279(52): 54952-62.

C. Results

1. *chx21chx23* pollen is not slow, but is infertile

Aim: To confirm that *chx21chx23* pollen is infertile.

Rationale: If *chx21chx23* pollen is just slow, but still fertile, then when 35, 70 and 140 of mixed *chx21* and *chx21chx23* pollen grains were used, same number of seeds would be produce compared to that of *chx21* single mutant or wt pollen, respectively. However, if *chx21chx23* pollen is infertile, then when 35 and 70 mixed pollen grains were used, less seeds would be produced compared to that of chx21 single mutant or wt pollen, respectively. When 140 pollen grains were used, mixed pollen grains, *chx21* single mutant and Wt pollen would produce the same amount of seeds as pollen became saturated. Moreover, when 70 pollen grains were used, mixed pollen would produce same amount of seeds as that of 35 chx21 single mutant or Wt pollen, as half of the mixed pollen were *chx21* single mutants (Fig. A).

Materials & methods: Were the same as that described in Chapter II and the Appendix. Each experiment was run three replicates.

Results: Mixed pollen produced less seeds than *chx21* and wt pollen when 35 and 70 pollen were used, respectively (Fig. B). The three different pollen populations produced about same amount of seeds when 70 pollen were used. Mixed pollen produced same amount of seed as 35 *chx21* and wt pollen, respectively. So, *chx21chx23* pollen is infertile.

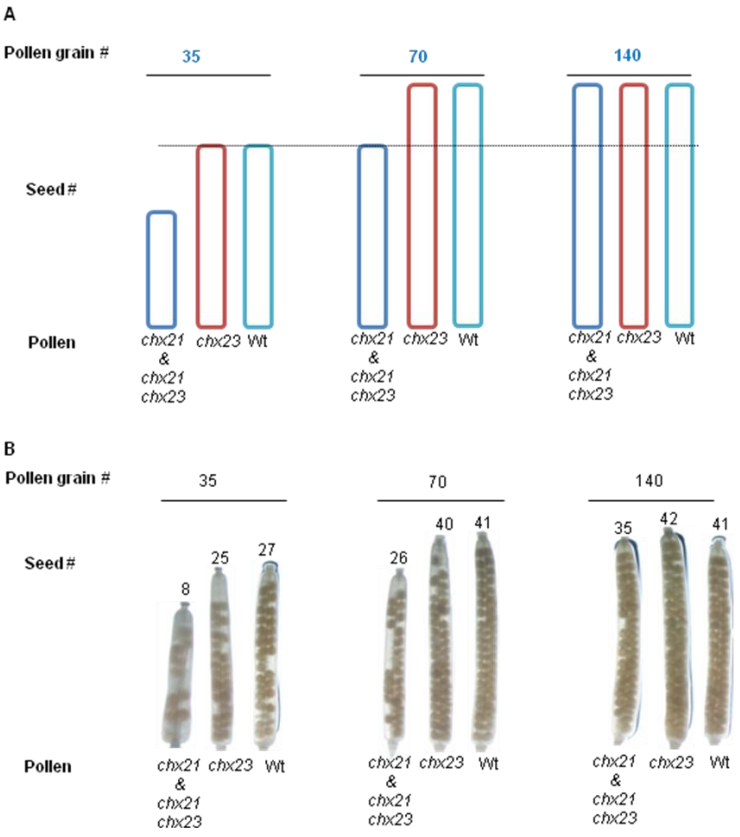


Figure VI-1. *chx21chx23* double mutant pollen is infertile. (A) Diagram of the rationale. (B) Seed pods and seeds number from fertilization using different pollen.

2. Yeast cell growth was not affected by flower proteins

Aim: To test if proteins from ovules activate CHX23 and enhance yeast cell growth.
Rationale/hypothesis: Unlike CHXs tested so far, CHX23 failed to show activity in yeast cells. Considering CHX23 play a role in pollen tube reorientation in response to guiding signal (s) from ovule/embryo sac, my hypotheses were: 1) a CHX23 activator was missed in the previous yeast assay; 2) yeast cells may have the same signal sensing and transducing mechanisms as Arabidopsis. So, with the presence of proteins isolated from female tissue, CHX23 expressed in yeast cells may get activated and affect cell growth.
Materials & Methods:

1) Flower protein isolation:
   - Collect 0.35 g ms-1 flowers (stage 13) and put into liquid N_2.
   - Drill & then add 350 µl extraction buffer, continue drilling till material is well homogenized.
   - Centrifuge @ 14000 rpm (16000 RCF) for 15 min @ 4 Cº.
   - Transfer the supernatant to a new tube and repeat step 3.
   - Transfer the supernatant to a new tube.
   - Filter sterilization.
   - Aliquot 100 µl to new tubes and put into -80Cº.
2) Protein concentration determination using the Bradford standard curve method.
3) Yeast growth test: the same as described in the Appendix protocols.

Extraction buffer:

Chemicals	Stock conc.	Final conc.	Vol/40 ml
Hepes_BTP, pH 7.4	1 M	50 mM	2 ml
D-Sorbitol	1 M	250 mM	10
BSA (0.5% W/V)			0.2 g
NP-40 (0.1%)			0.04 ml
PMSF	1 M	1 mM	40 µl
DTT	1 M	1 mM	40 µl
TPCK	0.2 M	0.1 mM	20 µl
Benzamidine	1.25 M	2 mM	64 µl
Leupeptin	1 mg/ml	1 µg/ml	40 µl
Pepstain	5 mg/ml	5 µg/ml	40 µl
H_2O			27.72 ml

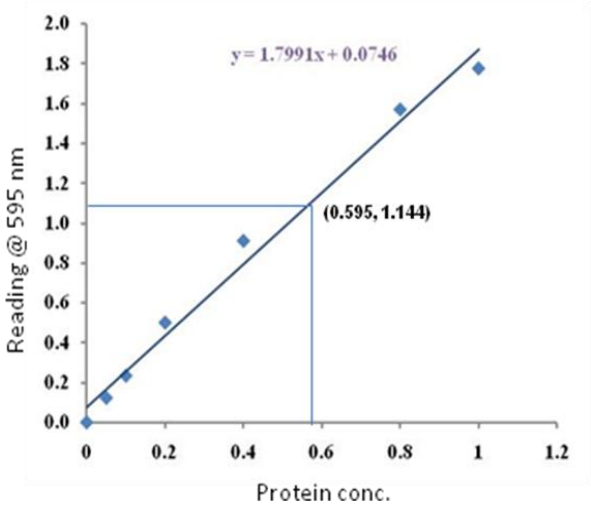
Results

Use the Bradford standard carve constructed by myself, I tested the protein concentration. 20 times diluted protein solution showed an OD reading value of 1.144, corresponding to 0.595 mg/ml concentration (Fig. A).

Different amount of proteins were added to 20 µl yeast cells that transformed with empty vector or CHX23 and then spotted on growth plates. In the treatment with 20 µg proteins, CHX23-transformed cells grew significantly better than empty vector-transformed cells (Fig. B).

Unfortunately, this result could not be repeated in following experiments (3 times)

A



B

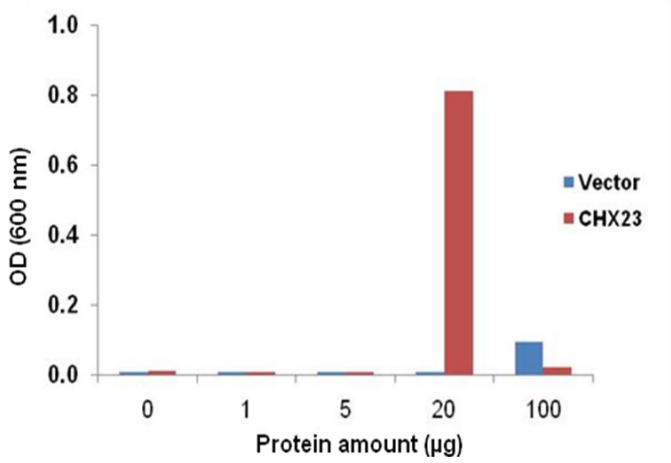


Figure VI-2. Effect of flower proteins on yeast cell growth. (A) Bradford standard curve and the protein concentration of the sample. (B) Flower proteins enhanced growth of yeast cells transformed with CHX23.

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Curriculum Vitae

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Education

09/1996 - 07/2000 **Bachelor of Science**. Qingdao Agricultural University (formerly Laiyang Agricultural College), Shandong, China.

09/2000 - 07/2003 **Master of Science.** China Agricultural University, Beijing, China.
Advisor: Dr. Chunjian Li
Thesis title: Effect of nitrogen on growth, nutrient uptake and distribution, and quality of flue-cured tobacco plants.

08/2004 – 05/2010 **Ph.D.** University of Maryland, College Park.
Advisor: Dr. Heven Sze
Dissertation title: Pollen tubes fail to target ovule in the absence of two cation/proton exchangers.

Professional experience

Research

2000-2003 Graduate research assistant, China Agricultural University, Beijing, China. Using quartz culturing combined with field experiment, I studied the effect of nitrogen on growth, nutrient uptake and distribution, and quality of flue-cured tobacco plants. My research resulted in four peer-reviewed papers.

2004-2010 Graduate Research Assistant, University of Maryland at College park. Identified the first transporter that plays key role (s) in pollen tube guidance in Arabidopsis. This study is to be submitted to Genes & Development.

Teaching & Mentoring

2005 Teaching Assistant, Plant Physiology (BSCI 442) laboratory. Univ MD

2006 Teaching Assistant, Cell Biology (BSCI 421) laboratory. Univ MD

2005-2010 Research mentor for 2 undergraduate and 3 high school students in the lab.

Honors

1. Marsho Award (The Best Graduate Student Presentation at the Annual Meeting, Mid- Atlantic Section of American Society of Plant Biologists). 2009
2. USDA (Department of Agriculture, U.S.A)-sponsored travel award for the Frontiers of Sexual Plant Reproduction III (FSPRIII) Conference at Tucson, Arizona. 2008.
3. Jacob K. Goldhaber Travel Grant. Graduate School, University of Maryland. 2008.
4. College-sponsored travel award for the 18th International Conference on Arabidopsis Research, Beijing, China. College of Chemical and Life Sciences, University of Maryland. 2007.
5. An Li-Fang Scholarship. China Agricultural University. 2003.

Professional Association

The American Society of Plant Biologists

Presentations on national and international meetings:

1. Annual Meeting of the American Society of Plant Biologists. *Cation/Proton Transporters are Key Players in Pollen Tube Guidance***.** Honolulu, Hawaii 2009. (I was the only graduate student speaker on Minisymposium 5: Reproductive Biology)
2. Society for Developmental Biology, Mid-Atlantic Regional Meeting. *Two Cation/Proton Transporters are Key Players in the Plant Reproductive Dialogue*. College Park, MD 2009 (Selected talk)
3. Annual Meeting, Mid-Atlantic Section of the American Society of Plant Biologists. *Cation/Proton Transporters are Key Players in Pollen Tube Guidance*. College Park, MD 2009 (Talk, The Best Graduate Student Presentation Award).
4. Frontiers of Sexual Plant Reproduction III (FSPR III) meeting. *Two Cation/Proton Exchangers are Involved in Pollen Tube Guidance*. Tucson AZ 2008. (Poster presentation)
5. The 18th International Conference on Arabidopsis Research. *Genetic Evidence for a Role of Cation/H+ Transporters in Plant Male Fertility*. Beijing, China 2007. (Poster presentation)
6. Annual Meeting, Mid-Atlantic Section of the American Society of Plant Biologists. *Genetic Evidence for a Role of Cation/H^+ Transporters in Plant Male Fertility*. College Park, MD. 2007. (Talk)

Peer reviewed papers (in chronological order):

1. **Lu Y.X.**, Wang G.Y., Jiang F. and Li C.J. (2003) A method for study nutrient cycling and recycling in higher plants. Plant Physiology Communications, 39(4): 359-362.
2. **Lu Y.X.**, Zhao Z.X., Li C.J., Zhang F.S. and Jiang R.F. (2004) Using plant nitrate concentration in fresh leaf tissues to predict the final quality of tobacco in Yunnan province. Scientia Agricultura Sinica 37: 68-72.
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4. Li C.J, Zhang F.S., Li W.Q., Zhao Z.X., Xi X.Y., Shi Q.M. Chao F.C., **Lu Y.X.**, Tao F. Qin Y.Q., Ju X.T. and Jiang R.F. (2007) Nitrogen management and its relation to leaf quality in production of flue-cured tobacco in China. Plant Nutrition and Fertilizer Science. 13(2): 331-337.
5. **Lu Y.X**., Zulkifli L., Johnson M., Palanivelu R., Uozumi N., Cheung A. and Sze H. (2010). Pollen tube fails to target ovule in the absence of two cation/proton exchangers in Arabidopsis. (In preparation).