Final report
2007-2016

Grant no. DEFG02-07ER15883

Title	**Endomembrane Cation Transporters and Membrane Trafficking**
Report period	2007-2016
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Sponsor's Number	DEFG02-07ER15883
Duration:	09/01/2007 – 01-31/2017

This brief report includes work conducted from Sept. 2007-Jan 31, 2017.

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Signature of Principal Investigator April, 2017

Abstract

Multicellular, as well as unicellular, organisms have evolved mechanisms to regulate ion and pH homeostasis in response to developmental cues and to a changing environment. The working hypothesis is that the balance of fluxes mediated by diverse transporters at the plasma membrane and in subcellular organelles determines ionic cellular distribution, which is critical for maintenance of membrane potential, pH control, osmolality, transport of nutrients, and protein activity. An emerging theme in plant biology is that cells respond and adapt to diverse cues through changes of the dynamic endomembrane system. Yet we know very little about the transporters that might influence the operation of the secretory system in plants. Here we focus on transporters that influence alkali cation and pH homeostasis, mainly in the endomembrane/ secretory system. The endomembrane system of eukaryote cells serves several major functions: i) sort cargo (e.g. enzymes, transporters or receptors) to specific destinations, ii) modulate the protein and lipid composition of membrane domains through remodeling, and iii) determine and alter the properties of the cell wall through synthesis and remodeling.

We had uncovered a novel family of predicted cation/H^+ exchangers (CHX) and K^+ efflux antiporters (KEA) that are prevalent in higher plants, but rare in metazoans. We combined phylogenetic and transcriptomic analyses with molecular genetic, cell biological and biochemical studies, and have published the first reports on functions of plant CHXs and KEAs. CHX studied to date act at the endomembrane system where their actions are distinct from the better-studied NHX (Na/K-H^+ exchangers). Arabidopsis thaliana CHX20 in guard cells modulate stomatal opening, and thus is significant for vegetative success. Other CHXs ensure reproductive success on dry land, as they participate in organizing pollen walls, targeting of pollen tubes to the ovule or promoting fertilization. Based on localization and mutant analyses, we conclude that CHXs modulate the ion balance, pH or both in micro-regions of endoplasmic reticulum, endosomes and prevacuolar compartment (PVC), and so influence membrane trafficking and signaling resulting in proper osmoregulation in guard cells and seed formation. We also demonstrated for the first time that AtKEA2 associates with chloroplasts, especially at the two poles of developing plastids. These results show that AtKEA1 and AtKEA2 transporters in specific microdomains of the inner envelope link local osmotic, ionic, and pH homeostasis to plastid division and thylakoid membrane formation. The first 3-D structure model of AtCHX was generated, and architecture-directed mutagenesis identified critical residues of the transport core giving insights to the transport mode of this family. Thus we have revealed for the first time crucial roles of an unknown K^+/H^+ transport family on plant growth (KEA), gas exchange, pollen cell wall, and different phases of reproduction (CHXs). The dynamic endomembrane of plant cells is integral to cytokinesis, cell expansion, defense, and cell wall formation, thus these studies are directly relevant to the mission of the Department of Energy and to a better understanding of determinants for enhancing plant biomass and plant tolerance to abiotic stress.

The Sze laboratory had a long-term interest in the regulation of energy-driven H^+ pumps and Ca-pumps in plants. pH and [Ca] changes in plants were recognized to be important as signaling intermediates in the regulation of plant development, growth and stress tolerance; however the molecular identity of any plant transporters was unknown in the 1980s. To identify the diverse array of transporters, the Sze laboratory developed *in vitro* methods to assay for ATP-driven H^+-pumps and ATP-driven Ca pumps, and these assays led to the purification and molecular identification of the vacuolar H+-ATPase complex (Sze 1984; Sze et al. 1995; Sze et al 2001) and two types of Ca-ATPases (Sze et al. 2000. DOE-supported). After the first plant genome was sequenced, Sze and coworkers discovered a large family of predicted cation/H^+ exchangers in the Arabidopsis thaliana var. Col. To understand the roles of this unknown transport family, the Sze laboratory first utilized bioinformatics, and transcriptomic approaches to provide clues and working ideas.

A. PROPOSED RESEARCH- SPECIFIC AIMS

I.-1. Determine the activity and regulation of selected CHXs by expression of full-length protein in yeast mutants.

I-2. Determine role of CHXs on pH_{cyt} and pH_{vac} in yeast using pH-sensitive dyes & reporters.

I-3. Test the potential regulatory role of the hydrophilic C tail of CHX.

I-4. Test in vivo functions of CHX and interactors using mutant plants & dominant negative constructs.

I-5. If time permits, determine membrane locations of CHX using confocal microscopy and EM, and test their roles on pH_{cyt} and $pH_{vac/lumen}$ in plant cells.

II-1. determine the transport mode of CHX which has been ambiguous so far.
II-2. test whether the hydrophilic carboxylic domain of CHXs might regulate activity, membrane sorting or both by truncations and point mutations
II-3. Find soluble and membrane interactors that give clues of potential regulators and effectors,
II-4. test the biological role of selected CHX proteins in plant cells.

We have accomplished most of the proposed goals, and made progress towards other aims. We were unable to make significant progress on the search for potential regulatory partners (aim I-4 or II-3).

B. RESULTS- Publications

1. Proliferation of a novel cation/H antiporter (CPA2) family in land plants

Chanroj S, GY Wang, K Venema, MW Zhang, CF Delwiche, H Sze (2012) Conserved and diversified gene families of monovalent cation/H+ antiporters from algae to flowering plants. ***Frontiers in Plant Science*** 3(25): doi 10.3389/fpls.2012.00025).

The discovery of a previously unrecognized family of cation/H antiporters in 2000 raised questions about their purpose and function. With no information about their function, we conducted a phylogenetic study to determine the ancestral origin of two separate plant CPA1 and CPA2 families. CPA2 includes CHX (cation/H exchanger) and KEA (K efflux antiporter) members. Phylogeny analysis showed CHX increased from 3-4 genes in early land plants to over 40 in eudicots (Chanroj et al. 2012). In contrast, the better-studied NHX members in the CPA1 family remained about the same in gene number (6 to 8) from moss to Medicago.

Furthermore, NHX members fall into 3 clades, a trait that is strictly conserved from algae to flowering plants. Results suggest that general functions of NHX are conserved across the plant kingdom. KEA genes are conserved from green algae to flowering plants, and their presence in red algae and secondary endosymbionts suggest a role in plastids. However, the diversity of CHX genes suggests they could function in adaptation to life on dry land through roles in vegetative survival, reproductive innovations or both.

2. **CHX or NHX share similar protein architecture and transport core**.

Czerny DD, Padmanaban S, Anishkin A, Venema K, Riaz Z, Sze H*. (2016) Protein architecture and core residues in unwound α-helices provide insights to the transport function of plant AtCHX17. Biochim Biophys Acta. 1858(9):1983-98.

Fig. 1. Predicted 3D structure of AtCHX17

So far, the transport activity of any CHX has not been directly demonstrated in vitro. To get insights to its transport mode, we combined structural modeling and mutagenesis to identify core residues. Using different programs, the 3D model of AtCHX17 predicts a protein fold similar to E. coli NhaA or T. thermophilus NapA (Czerny et al 2016). The unique architecture of these transporters consists of two transmembrane helices (TM4 and TM11) that cross over at the short unwound region in the middle of the membrane. Residues in the discontinuous regions (T170 and K383) are in close proximity to a conserved aspartate residue (D201) in the middle of TM5. Mutagenesis of this aspartate and residues in the unwound helices provided insights that AtCHX17 has a transport core similar to characterized cation/H+ exchangers. CHXs appear distinct from NHX1, in having a critical glutamate residue (E111) at the end of TM2, near the membrane surface. Mutation of E111 inhibited CHX17 activity suggesting it has a role in pH sensing. CHX 17 activity was monitored by its ability to confer resistance of yeast mutants to Hygromycin B (see next section). These findings suggest that CHX and NHX share similar transport activity, but may differ in their regulation by pH. Future mutagenesis combined with direct transport assays are needed to shed more light on the mode of transport and the residues forming the active core.

3. **Differential activities of NHX and CHX in yeast.**

Chanroj S, YX Lu, S Padmanaban, K Nanatani,N Uozumi, R Rao, H Sze (2011) Plant-specific cation/H+ exchanger 17 and its homologs are endomembrane K+ transporters with roles in protein sorting. ***J Biol Chem*** 286(39):33931-41

Despite similarity between NHX and CHX proteins, the first clue they may differ in function came from studies using yeast. The yeast mutants lacked either orthologous genes in yeast or additional cation handling transporters: i) Plant NHX1 conferred yeast tolerance to high external levels (e.g. 0.5 M) of K or Na at acidic pH, whereas AtCHX17 or AtCHX20 were incompetent; (ii) AtCHX17 conferred tolerance to grow on alkaline medium with low K levels, however, AtNHX1 was ineffective (Chanroj et al. 2011); and (iii) although both conferred resistance to HygB at pH 5.6, only AtNHX1 restored full growth. In addition, yeast mutants containing a wild-type ScNhx1 but lacking K+ uptake transporters, Sctrk1trk2, failed to grow on medium containing low K+, while growth was restored with AtCHX17. These findings clearly underscore AtCHX17 perform different roles from AtNHX1 in the unicellular model yeast, though there is partial overlap in conferring HygB resistance between AtCHX17 and NHX1. Notably, the differential activity seen at acidic versus alkaline external pH would suggest that both participate in pH homeostasis; though AtNHX1 is active at acidic pH whereas AtCHX17 is active at alkaline pH. These results are consistent with yeast ScNhx1 and ScKha1 which are activated at pH 5 (Nass R et al., 1997. J Biol Chem 272:26145) and at weak alkaline pH (Wu X et al. 2016. J Biol Chem 291:9796), respectively.

Table 1**. Differential Activities of plant NHX and CHX expressed in yeast**

Transporter	Localization	Growth Activity in Yeast			
Plant or Yeast (Sc)	Yeast	**I.High K /Na, pH ~5**	**II.Low K at pH 7.5**	**III.low K+ & pH 7.5**	**IV.Hyg B at pH 5.6**
NHX5, NHX6	endomemb	Yes	n.d.		Yes (+++)[a]
NHX1, NHX2	endomemb	Yes (+++)	no	No	Yes (+++)
ScNhx1	PVC (yeast)	Yes[c]	no		Yes (+++)
AtCHX17, 18, 19	endomemb	no	yes (+++)	yes (+++)	yes (+)
AtCHX20	endomemb	No[b]	yes (++)	no	no
ScKha1	Golgi (yeast)		Yes (+++)	Yes (+++)	Yes (++)

I. Yeast strain KTA40-2, growth tested at pH 5.
II, IV. Yeast strain used was KTA40-2, unless otherwise indicated.
III. Yeast used was LMM04 (Nhx1, trk1,2, tok1).
Padmanaban et al. 2007; Chanroj et al. 2011; Bassil et al. 2011.

4. Effect of plant CHX17 on vacuolar biogenesis and protein sorting in yeast

Chanroj S, YX Lu, S Padmanaban, K Nanatani,N Uozumi, R Rao, H Sze (2011) Plant-specific cation/H+ exchanger 17 and its homologs are endomembrane K+ transporters with roles in protein sorting. ***J Biol Chem*** 286(39):33931-41

Wild-type yeast cells grow well on either acidic (pH 5-6) or weakly alkaline (pH 7.5) medium. Yet some mutants continue to proliferate at pH 6, though not at alkaline pH, suggesting that specific cellular processes are compromised when environment pH becomes alkaline. Genome wide screens of mutants unable to growth at alkaline pH highlight genes involved in V-ATPase activity, vacuolar biogenesis and protein sorting. Thus the ability of AtCHX17 to restore growth at weakly alkaline pH in yeast mutants lacking several cation transporters suggests CHX17 has a role in pH homeostasis and possibly vacuole biogenesis.

This idea was confirmed when we showed that i) CHX17 conferred tolerance to HygB an antibiotic that is endocytosed and sorted to the vacuole for inactivation, and ii) expression of CHX17 in mutant lacking cation transporters, reduced secretion of a vacuole-destined enzyme, CPY (Chanroj et al., 2011). Together these results suggest that CHX17 activity affects vacuole membrane trafficking and protein sorting of a soluble vacuolar enzyme.

5. CHX genes affect male fertility at several phases of reproduction

Nearly 20 AtCHX genes are specifically or preferentially expressed in the male gametophyte (Sze et al., 2004; Bock et al. 2006). Transcriptome analyses showed that some genes are expressed during development of the pollen grain, while others are expressed in pollen tubes after germination on stigmas (see Pollen Navigator website: pollen.umd.edu). Genes induced by *in vivo* pollen germination suggests roles in post-pollination events. Predicted CHX orthologs from many flowering plants fall into similar subclades as Arabidopsis CHX (Chanroj et al. 2012), underscoring the relevance of this gene family to all plants.

a) K^+ transporters, CHX21 and CHX23, facilitate targeting of pollen tubes to the ovule

Lu YX, S Chanroj, L Zulkifli, M Johnson, N Uozumi, AY Cheung, H Sze* (2011). Pollen tubes lacking a pair of cation/H^+ exchangers fail to target ovules in Arabidopsis. Plant Cell 23(1):81-93]

Male fertility was severely impaired in *chx21chx23* double mutants, but not in single *chx21* or *chx23* mutants. Double mutant pollen grains were able to germinate, and pollen tubes grew through the entire length of the transmitting tract. Oddly, *chx21chx23* mutant tubes failed to turn towards the ovules and did not enter the micropyle (Lu et al. 2011). CHX23 can mediate K transport, as shown by its ability to restore growth of E coli lacking 3 K^+ uptake systems. The fluorescent protein-tagged CHX23 was co-localized with reticulate membranes and endoplasmic reticulum markers in transiently transfected pollen tubes. The possibility that active CHX23 is localized to specific ER domains or to other mobile membranes that interact with the ER, remains to be examined. Based on these findings, we proposed that the modulation of localized cation and pH balance by CHX21 and CHX23 could influence either signal reception, signal transduction or both that lead to a shift in directional tube growth towards the ovule (Lu et al. 2011).

b) CHX17, CHX18 and CHX19 affect pollen wall formation, male fertility and embryo development

Padmanaban S, Czerny DD, Levin KA, Leydon AR, Su RT, Maugel TK, Zou Y, Chanroj S, Cheung AY, Johnson MA, Sze H. (2017) Transporters involved in pH and K+ homeostasis affect pollen wall formation, male fertility, and embryo development. J Exp Bot. Feb 23. doi: 10.1093/jxb/erw483

AtCHX17 shared high similarity to AtCHX18 and to AtCHX19 in protein sequence, functional activity in yeast and in membrane localization (Table 1). The first morphological phenotype was observed in the triple *chx17chx18chx19* mutant, whereas changes were not detected in single or double mutants (Chanroj et al. 2013). The results implied one gene can substitute for loss-of-function of a sibling gene even though the two genes displayed differential tissue expression in the wild-type plant.

The vegetative growth and flower development of the triple c*hx17chx18chx19* mutant was similar to those of the wild-type, but seed set was reduced by 50-70%. Mutant pollen tubes grow, target, and enter ovules; however many targeted ovules fail to develop into seeds. Live cell-imaging to monitor fertilization indicated that failed fertilization could be due to impaired pollen tube or sperm function (Padmanaban et al. 2017). AtCHX19 and AtCHX18 are expressed in the tube and sperm cell, respectively. The highly reticulate pattern of pollen grain walls was disorganized in the triple mutant. AtCHX17 is expressed early in grain development at the microspore and bicellular stage (Bock et al. 2006) when pollen walls are formed. Although the wall phenotype alone is unlikely to cause reduced fertility, results suggest that processes involved in wall modeling are likely compromised in chx mutants. As pH and cation homeostasis mediated by AtCHX17 affects membrane trafficking and cargo delivery (Chanroj et al 2011), these results infer that pollen tube and sperm function are dependent on proper cargo sorting and secretion that remodel walls and components of the plasma membrane.

To my knowledge, these are the first reports to show reproductive defects in higher-order *chx* mutants. These publications demonstrate definitive roles of pH and cation homeostasis at diverse phases of reproduction. The roles of many other CHXs remain to be uncovered.

6. K^+/H^+ antiporters (KEA1,2) on chloroplast function and development

(Collaboration with Kees Venema lab in Spain)

*Aranda-Sicilia MN, Aboukila A, Armbruster U, Cagnac O, Schumann T, Kunz HH, Jahns P, Rodríguez-Rosales MP, Sze H, Venema K. (2016) Envelope K+/H+ antiporters AtKEA1 and AtKEA2 function in plastid development. Plant Physiol. 172(1):441-9. doi: 10.1104. (collaboration with Venema lab)

*Aranda-Sicilia MN, Cagnac O, Chanroj S, Sze H, Rodríguez-Rosales MP, Venema K. (2012) Arabidopsis KEA2, a homolog of bacterial KefC, encodes a K(+)/H(+) antiporter with a chloroplast transit peptide. ***Biochim Biophys Acta***. 1818(9):2362-71 (This paper resulted from collaboration with the Venema lab.)

Photosynthesis drives life as we know it on this planet, yet the roles of K+ transporters on chloroplast development and function are largely unknown. In a collaboration with the Venema laboratory, we provided the first *in vitro* evidence that Arabidopsis KEA2 is a K^+/H^+ antiporter. Furthermore the N-terminus of KEA2 targets to the plastid (Aranda-Sicilia et al. 2012). Subsequently our findings were extended and AtKEA1 and AtKEA2 were shown to localize at the inner envelope. Moreover, loss of function of these two genes led to reduced photosynthesis efficiency and plant growth (Kunz HH et al. 2014. Proc Natl Acad Sci 111:7480-5) suggesting a critical role for K^+/H^+ homeostasis. However, fluorescent tag-labeled AtKEA2 localized to the caps of small, dividing plastids of young expanding leaves. Double mutants contained less plastid per cell. Together, the results show that K^+/H^+ antiporters at the inner envelope link local pH and cation homeostasis to plastid division and thylakoid membrane formation.

To our knowledge, these constitute the first studies to define the functions of previously unknown K+ transporters in chloroplast development and photosynthetic efficiency.

7. Endomembrane transporter AtCHX20 affects guard cell movement.

Only one CHX gene is preferentially expressed in Arabidopsis guard cells, though orthologs of AtCHX20 are present in early land as well as flowering plant. Thus its cellular function is likely conserved in all plants. Single mutants showed a 35% reduction in light-induced stomatal opening compared to wild-type plants (Padmanaban et al, 2007). The results are consistent with the idea that cation and pH homeostasis of particular endomembrane by CHX20 is critical for dynamic membrane trafficking as guard cells osmoregulate to open the stomatal pore.

Significance

We have accomplished most of the proposed goals, and made progress towards other aims. We were unable to make significant progress on the search for potential regulatory partners (aim I-4 or II-3). Support from DOE resulted in ten refereed publications.

We revealed for the first time crucial roles of an unknown transport family in plant growth (KEA1,2), gas exchange (CHX20), pollen targeting to ovules and fertilization (CHX17, CHX21,23). Structural modeling and mutagenesis support the idea that CHXs mediate K/H exchange. Our studies support the idea that K^+/H^+ exchangers are able to affect diverse biological processes through cation and pH homeostasis at diverse intracellular membranes and organelles. As the endomembrane system of eukaryote cells serves major functions in sorting cargo (e.g. enzymes, transporters or receptors) to specific destinations, and in determining properties of the cell wall through synthesis and remodeling, the transporters are intimately linked to signaling, development and tolerance to stress. Future studies of CHX transporters in energy or crop plants promise to reveal strategies that plants use for adaptation to life on dry land through vegetative survival, reproductive innovations or both.

Publications (*supported in part by DOE)

*Aranda-Sicilia MN, Aboukila A, Armbruster U, Cagnac O, Schumann T, Kunz HH, Jahns P, Rodríguez-Rosales MP, Sze H, Venema K. (2016) Envelope K+/H+ antiporters AtKEA1 and AtKEA2 function in plastid development. Plant Physiol. 172(1):441-9. doi: 10.1104. (collaboration with Venema lab)

*Aranda-Sicilia MN, Cagnac O, Chanroj S, Sze H, Rodríguez-Rosales MP, Venema K. (2012) Arabidopsis KEA2, a homolog of bacterial KefC, encodes a K(+)/H(+) antiporter with a chloroplast transit peptide. ***Biochim Biophys Acta***. 1818(9):2362-71 (This paper resulted from collaboration with the Venema lab.)

*Bock KW, D Honys, JM. Ward, S Padmanaban, EP Nawrocki, KD Hirschi, D Twell, and H Sze8 (2006) Integrating Membrane Transport with Male Gametophyte Development and Function through Transcriptomics. **Plant Physiol**. 140, 1151-1168.

*Chanroj S, GY Wang, K Venema, MW Zhang, CF Delwiche, H Sze (2012) Conserved and diversified gene families of monovalent cation/H+ antiporters from algae to flowering plants. ***Frontiers in Plant Science*** 3(25): doi 10.3389/fpls.2012.00025).

***Chanroj S**, Padmanaban S, Czerny DD, Jauh GY, **Sze H**. (2013) K+ transporter AtCHX17 with its hydrophilic C tail localizes to dynamic membranes of the secretory/endocytic system: role in reproduction and seed set. Mol. Plant 6(4):1226-46.

*Chanroj S, YX Lu, S Padmanaban, K Nanatani,N Uozumi, R Rao, H Sze (2011) Plant-specific cation/H+ exchanger 17 and its homologs are endomembrane K+ transporters with roles in protein sorting. ***J Biol Chem*** 286(39):33931-41 [pdf sent]

Czerny DD, Padmanaban S, Anishkin A, Venema K, Riaz Z, Sze H. (2016) Protein architecture and core residues in unwound α-helices provide insights to the transport

function of plant AtCHX17. Biochim Biophys Acta. 1858(9):1983-98. doi: 10.1016/j.bbamem.2016.05.008. PubMed PMID: 27179641

Gwangbae Bak, Eun-Jung Lee, Yuree Lee, Mariko Kato, Shoji Segami, Heven Sze, Masayoshi Maeshima, Jae-Ung Hwang, and Youngsook Lee (2013). Rapid structural changes and acidification of guard cell vacuoles during stomatal closure require phosphatidylinositol 3,5-bisphosphate. The Plant Cell 25(6):2202-16.
Highlighted in **Faculty of 1000** (Plant Biology) by two Faculty Members

Lu YX, S Chanroj, L Zulkifli, M Johnson, N Uozumi, AY Cheung, H Sze (2011). Pollen tubes lacking a pair of cation/H^+ exchangers fail to target ovules in Arabidopsis. Plant Cell 23(1):81-93] PMID: 21239645.
Highlighted in Faculty of 1000 (Plant Biology) by: Wolf B Frommer Carnegie Institution, USA, Jan 2011

Padmanaban S, Chanroj S, Kwak J, Li X, Ward JM, Sze H. (2007) Participation of an endomembrane cation/H^+ exchanger AtCHX20 in osmoregulation of guard cells. **Plant Physiol**. 144(1): 82-93.

*Padmanaban S, Czerny DD, Levin KA, Leydon AR, Su RT, Maugel TK, Zou Y, Chanroj S, Cheung AY, Johnson MA, Sze H. (2017) Transporters involved in pH and K+ homeostasis affect pollen wall formation, male fertility, and embryo development. J Exp Bot. 2017 Feb 23. doi: 10.1093/jxb/erw483

Zhao J, Cheng NH, Motes CM, Blancaflor EB, Moore M, Gonzales N, Padmanaban S, Sze H, Ward JM, Hirschi KD. (2008) AtCHX13 is a Plasma Membrane K^+ Transporter. Plant Physiol. 148(2):796-807.

Website:

Pollen Navigator: pollen.umd.edu.

Search expression of any gene at seven different developmental stages from microspore to in vivo germinated pollen tubes.

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c) Mode of CHX transport

Rationale & Working hypothesis: The transport mode of CHX proteins is ambiguous. Our results show that CHX17 and CHX20 mediate K^+ transport after expression in E coli strain LB2003. CHX17 shows channel-like properties based on its similarity to KAT1, an inward-reticifying K+ channel, and CHX20 may be a H+-coupled transporter as it altered intracellular pH in yeast. The original plan to measure channel activity in giant spheroplast of CHX17 expressed in E coli yielded little to no current. Another approach is to use homology modeling and test CHX17 activity after site-directed mutagenesis. Here we report preliminary results that show this approach is working.

i) Homology Modeling of AtCHX17 with EcNhaA.

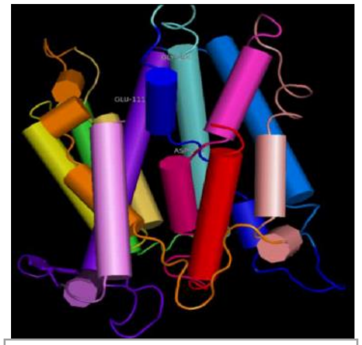

Fig. 1. Predicted 3D structure of AtCHX17

Chx 17 is a K^+ transporter that shares high homology to the E.coli protein NhaA (Chanroj et al. 2012). EcNhaA is a monovalent cation/H+ antiporter that has been successfully crystalized. The 3-D structure of EcNhaA coupled with mutagenesis studies has identified key residues important in the transport activity and regulation of the protein. We first generated a 3D model of *Arabidopsis thaliana* CHX17 (Fig. 1). Based on the linear alignment of EcNhaA and Chx17 and their 3D structural similarity, we identified candidate residues important in CHX17 function. These are Glu111 (TM2), Pro158 (L3), Asp201 (TM5), H284 (TM8), and Lys355 (TM10). Some of these residues were mutated by site-directed mutagenesis and mutated constructs were expressed in yeast and E coli mutants to test their activities.

ii) Testing activity of mutated CHX17 in yeast and in bacteria.

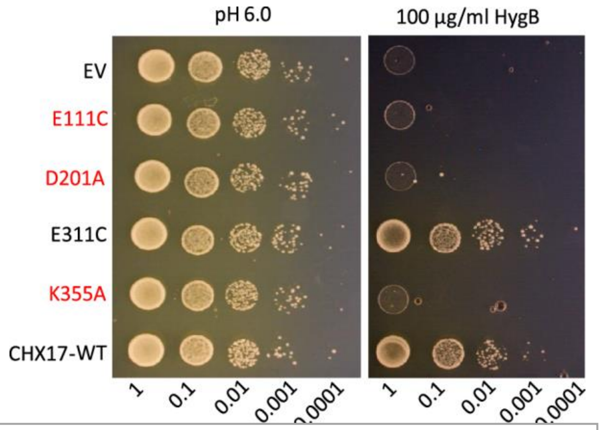

Fig. 2. KTA40-2 yeast (*ena1-4Δ, nha1Δ, nhx1Δ, kha1Δ*) expressing empty vector (EC) or CHX17 harboring single residue mutations are sensitive to hygromycin. WT-CHX17 restores yeast growth on Hyg.

Fig. 1 shows that CHX17 with mutations D201A, K355A or E111C were unable to restore growth of KTA-40-2 yeast on medium containing hygromycin similar to empty vector. Wild-type CHX17 restored growth. Results show that residues D201 and K355 are critical for activity, though E311 has little to no effect. As these residues are conserved in EcNhaA as D164 and K300, and NhaA is a Na/H+ exchanger, our results would support the model that CHX17 operates in cation/H+ exchange.

Surprisingly, when mutated CHX17 was expressed in a K+-uptake defective E. coli strain, it restored growth of bacteria in low K+- containing medium similar to the wild-type gene. This would indicate that the protein is not acting as cation/H+ exchange when it is expressed in the PM of a bacterial.

We plan to mutate more key residues to test whether CHX17 behaves like a cation/H antiporter and to reveal how it might differ from other well-studied transporters, like NHX1 and KEA2. We are also talking with protein modelers to help interpret the models and identify key residues.

d) **Role of the C tail of CHX17**.

CHX proteins consist of a hydrophilic domain at the carboxyl-terminus (Ct) that is much longer than that of NHX family. The amino acid sequence of the Ct region shares little similarity to other CPA2 proteins (e.g. ScKHA1) and nothing is known about the role of this hydrophilic domain in plant CHX or yeast KHA1. We want to test the role of this domain.

A ***working hypothesis*** is that the hydrophilic region is cytoplasmic and has regulatory roles. It may be involved in regulating transport activity, interaction with signaling molecules, or cell components that affect membrane trafficking and protein sorting. Although the transmembrane (TM) domains of CHX17 and CHX20 are relatively conserved, their C tails show regions of divergence. We proposed to make C tail (Ct) truncations and single residue mutations to begin testing their role in yeast or in E coli. Residues affecting potential phosphorylation will be mutated e.g. S->D or S->A.

Evidence for a role of the C tail in sorting is shown by mis-sorting of the truncated CHX17(1-472) (Chanroj et al. *Mol Plant* in press. Pdf attached). Full-length protein is localized to the PM and PVC (prevacuolar compartment), yet the truncated, partially active protein did not in transgenic plants. Results also suggest that the tail may regulate activity, as CHX17(1-472) is partially (30%) effective in mediating E coli growth when expressed in a strain lacking three K$^+$ uptake pathways. Strains harboring vector only grew poorly on low K$^+$-containing medium, whereas full length CHX17-enhanced growth (OD$_{600}$) (not shown).

To understand the role of C tail, we will identify conserved residues in the C tail (430-472) that might regulate activity and alter protein sorting. Then test the residues by site-directed mutagenesis on activity using yeast or E coli, and on membrane localization in plant cells.

e) Identifying regulators and interacting proteins. These studies are now on hold due to a new discovery. We have recently found the first biological role of CHX17, CHX18, and CHX19 genes.

f) **Roles in reproduction and seed development**.

The reduced frequency of triple mutant (chx17/18/19) and quadruple mutant (chx16/17/18/19) recovered suggested a defect in seed set. To test this, we analyzed seed pods. Unexpectedly, we found that pods from either triple or quadruple mutants showed only 30-50% seed set (Fig. 3 from Chanroj et al in press).

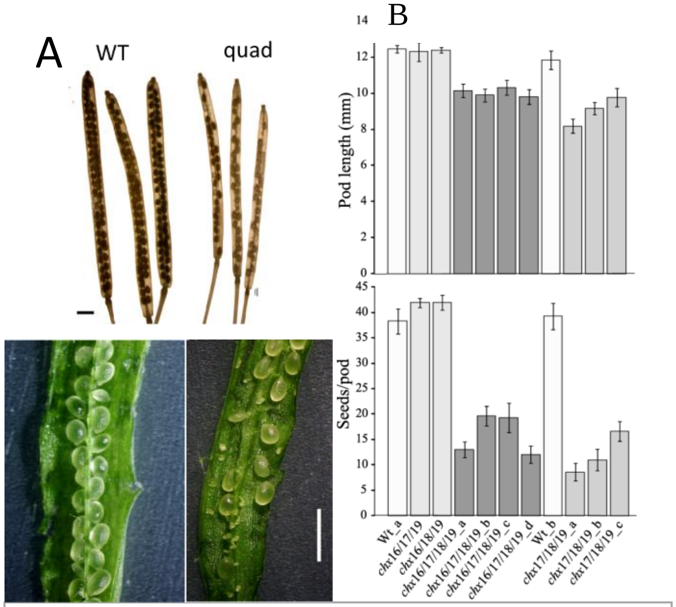

Fig. 4. A. Pods cleared of chlorophyll show less seeds in 4x mutant (quad) than in wild-type (top panel). Opened pods (bottom panel). B. Seed number per pod (bottom) of 3x and 4x mutant is lower than wild-type. Pod length varies a little (top).

This is the first evidence to show that several related CHX genes have a role in reproduction and/or seed set. This finding opens up new questions: Why? How? Where?

Plans for the coming year.

We did not see reduced seed set in double mutants, thus a combination of 3 genes, CHX17-19, seem to play the major role.

i. Is defect due to a male or female gametophyte malfunction? Testing this by manual pollination using either a heterozygous mutant as pollen donor to Wt stigma or vice versa.

ii. Is defect due to failed fertilization or aborted embryo development? Testing this by examination of ovule at different times after fertilization using DIC microscopy and clearing with chloral hydrate.

iii. Some pollen tubes clearly extend to the bottom of the ovary indicating pollen tube growth is not compromised. So we want to test if fertilization is compromised. We propose to visualize pollen discharge and fertilization in vivo using a sperm-specific promoter-driven histone-tagged to fluorescent protein.

iv. We are also working to test if a wild-type AtCHX17 gene and a mutated or truncated gene will rescue the mutant phenotype.

2. Anticipated unexpended funds by Aug. 31, 2013
$60,000

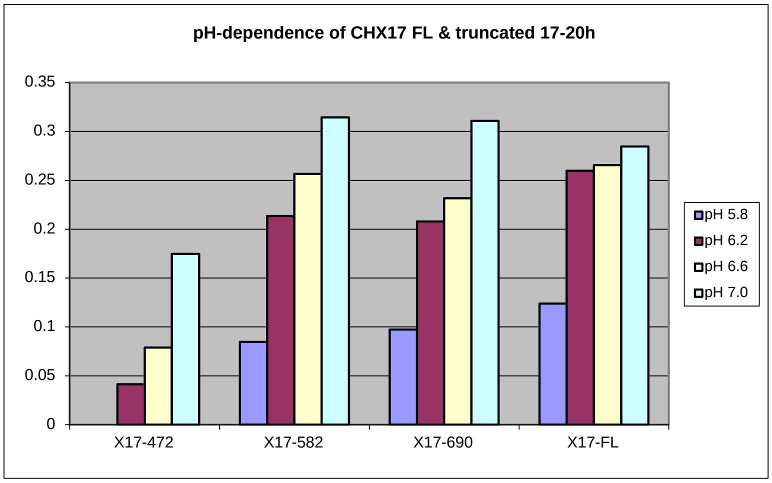

Fig. 2. C tail is important for activity. Growth of E coli strain LB2003 expressing various lengths of CHX17 at different medium pH. Full length (FL) and truncated CHX17 is shown on x axis.

Fig. 2 also shows that CHX17-582 containing 110 more residues of the C tail showed nearly similar activity as the full-length protein. However, CHX17-472 containing a very short C tail is impaired in growth at all pHs tested, suggesting that part of the C tail is important in modulating CHX activity.

We are checking whether protein expression in the truncated CHX17 is equivalent using anti-V5 epitope. We are currently searching for motifs that may contain residues critical for activity and for sorting. We are selecting potential candidates for mutagenesis.

ATTN: Continuation request for Grant No. DE-FG02-07ER15883