Final Report

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Title **Regulating Intracellular Calcium in Plants: From Molecular Genetics to Physiology**

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Heven Sze

Signature of Principal Investigator

June 22, 2008

Date

Note to Sze: submitted online 4 Plant Physiol papers to http://www.osti.gov/elink-2413.

Title: **Regulating Intracellular Calcium in Plants: From Molecular Genetics to Physiology**

Abstract

To grow, develop, adapt, and reproduce, plants have evolved mechanisms to regulate the uptake, translocation and sorting of calcium ions into different cells and subcellular compartments. Yet how plants accomplish this remarkable feat is still poorly understood. The spatial and temporal changes in intracellular [Ca^{2+}] during growth and during responses to hormonal and environmental stimuli indicate that Ca^{2+} influx and efflux transporters are diverse and tightly regulated in plants. The specific goals were to determine the biological roles of multiple Ca pumps (ECAs) in the model plant Arabidopsis thaliana. We had pioneered the use of K616 yeast strain to functionally express plant Ca pumps, and demonstrated two distinct types of Ca pumps in plants (Sze et al., 2000. Annu Rev Plant Biol. 51,433). ACA2 represented one type that was auto-inhibited by the N-terminal region and stimulated by calmodulin. ECA1 represented another type that was not sensitive to calmodulin and phylogenetically distinct from ACAs.

The goal to determine the biological roles of multiple ECA-type Ca pumps in Arabidopsis has been accomplished. Although we demonstrated ECA1 was a Ca pump by functional expression in yeast, the in vivo roles of ECAs was unclear. A few highlights are described. ECA1 and/or ECA4 are Ca/Mn pumps localized to the ER and are highly expressed in all cell types. Using homozygous T-DNA insertional mutants of eca1, we demonstrated that the ER-bound ECA1 supports growth and confers tolerance of plants growing on medium low in Ca or containing toxic levels of Mn. This is the first genetic study to determine the in vivo function of a Ca pump in plants. A phylogenetically distinct ECA3 is also a Ca/Mn pump that is localized to endosome, such as post-Golgi compartments. Although it is expressed at lower levels than ECA1, eca3 mutants are impaired in Ca-dependent root growth and in pollen tube elongation. Increased secretion of wall proteins in mutants suggests that Ca and Mn homeostasis in post-Golgi compartments are critical for secretory activities. Moreover, perturbation of the secretory machinery limits growth possibly by upsetting the synthesis, processing and assembly of cell wall components. Analyses of whole genome transcriptome of pollen shows that a subset of Ca pump genes are developmentally regulated. Each ECA Ca pump is localized to distinct endomembrane compartments and regulate Ca and Mn homeostasis required for optimal growth and for tolerance to high Mn stress. Ca and Mn levels within endomembrane lumen appear to be critical for activities of the secretory machinery including post-Golgi compartments that coordinate membrane traffic and sorting of materials to the vacuole and the cell wall. Significance: Thus sorting of Ca/Mn by ECA pumps in endomembranes is critical for membrane trafficking pattern which serves as a central coordinator of plant growth, development and adaptation to abiotic and biotic stress.

I. Introduction & Goals of the Project.

To grow, develop, adapt, and reproduce, plants have evolved mechanisms to regulate the uptake, translocation and sorting of calcium ions into different cells and subcellular compartments. Yet how plants accomplish this remarkable feat is still poorly understood. The spatial and temporal changes in intracellular [Ca^{2+}] during growth and during responses to hormonal and environmental stimuli indicate that Ca^{2+} influx and efflux transporters are diverse and tightly regulated in plants. The specific goals were to determine the biological roles of multiple Ca pumps (ECAs) in the model plant Arabidopsis thaliana. We had pioneered the use of K616 yeast strain to functionally express plant Ca pumps, and demonstrated two distinct types of Ca pumps in plants (Sze et al., 2000). ACA2 represented one type that was auto-

inhibited by the N-terminal region and stimulated by calmodulin. ECA1 represented another type that was not sensitive to calmodulin and phylogenetically distinct from ACAs.

One proposed goal to identify potential regulators of ECA1 Ca pump and define the functional and regulatory regions/residues of ECA1 by mutagenesis, has been sidelined for the present. We screened for suppression of pmr1 yeast on Ca-depleted medium after transforming ECA1-expressing yeast with an Arabidopsis cDNA library. Pmr1-expressing ECA1 normally are able to grow on medium depleted of Ca (+ EGTA). Although several interesting candidate regulators came out of the screen, the results were not robust or reproducible. The identity of several candidate proteins was hypothetical or unknown. So this goal was not pursued.

We were successful in accomplishing the second major goal to determine the biological roles of multiple ECA-type Ca pumps in Arabidopsis. Although we demonstrated ECA1 was a Ca pump by functional expression in yeast, the in vivo roles of ECAs was unclear. Highlights of our progress include the following:

- Using homozygous T-DNA insertional mutants of eca1, we demonstrated that the ER-bound ECA1 supports growth and confers tolerance of plants growing on medium low in Ca or containing toxic levels of Mn. This is the first genetic study to determine the in vivo function of a Ca pump in plants. Ca stored in the ER lumen is potentially important for Ca release and Ca oscillations in response to signals and for biochemical activities, thus the identification of an abundant Ca pump at the ER is significant for understanding how plants respond to biotic signals and to abiotic stress.
- A novel Ca pump, ECA3, was recently uncovered based on sensitivity to a specific inhibitor, thapsigargin, and localization to post-Golgi membranes. To my knowledge, this is the first endosomal Ca pump identified at the molecular level from plants.
- Using mutants of eca3, we find that ECA3 supports pollen tube elongation and primary root growth in a Ca-dependent manner. Increased secretion of wall proteins in mutants, suggested that Ca in the post-Golgi compartments supports growth by regulating secretory activities. Thus endosomal ECA3 appears to have a significant role in the synthesis, processing and assembly of cell walls.
- We have begun to integrate transport with the plant life-cycle using the male gametophyte as a model. Analysis of whole-genome transcriptome shows that a subset of Ca pump genes are expressed early in development whereas others appear late in mature pollen. These expression patterns provide clues and working ideas to test functions of specific transporters.

II. PROGRESS

Several papers have been published and another one is being revised for submission. The work has also been presented at seminars, meetings, and in posters at international meetings.

These and other major scientific contributions are listed below.

i) **ER-bound ECA1 supports growth and confers tolerance to low Ca and high Mn**. Wu et al 2002. Plant Physiol. 130:128-37

Arabidopsis contains 14 predicted Ca-ATPases, though the in vivo functions of each

were not known. We reported the first genetic study to determine the in vivo functions of a Ca pump in plants. We had shown before through expression in yeast mutant that ECA1 was a high-affinity Ca pump (Km 30 nM) (Liang et al. 1997; Liang & Sze, 98); however we found that ECA1 also transport other divalent cations. Evidence supporting this are (i) ECA1-expressing yeast K616 was tolerant to high Mn and Zn, (ii) Mn and Zn decreased the formation of Cadependent [^{32}P]phosphoprotein intermediate; (iii) Mn inhibited 45Ca uptake into vesicles from ECA1-expressing yeast. Homozygous Arabidopsis mutants showed four-fold less Ca-pumping activity indicating that ECA1 contributes to most of the CPA-sensitive Ca pump activity. Under optimal growth conditions (1.5 mM Ca and 50 µM Mn), eca1 mutants displayed little or no difference from Wt plants. However, when external [Ca] was depleted to 0.2 mM, mutants grew poorly. Furthermore, mutants grown on medium containing 500 µM Mn was stunted in root hair growth, chlorotic and reduced in fresh weight. These results show that despite the multiplicity of Ca transporters, pumping of Ca and Mn by ECA1 into the ER is required to support plant growth under standard conditions, and under conditions of Ca deficiency or Mn toxicity. The results are consistent with the idea that ECA1 supports growth in several ways, (i) load Ca and Mn into ER lumen for biochemical reactions, ii) lower [Ca]cyt, and iii) remove toxic levels of Mn from the cytosol (Wu et al. 2002).

Later studies using promoter::Gus showed that ECA1 is highly and widely expressed in many plant tissues relative to other 3 ECAs (Li et al., Plant Physiol. in press 6/08). Thus ECA1 seems to the most abundant ER-bound Ca pump of the ECA subfamily. As Ca levels in the ER lumen is important for Ca release during signaling, unfolded protein response, and other biochemical activities, the identification of this major ER Ca pump is relevant and significant for understanding how plant cells tolerate abiotic and biotic stress.

ii) An endomembrane Ca/Mn pump AtECA3 supports pollen tube elongation

(Li X et al. ms in revision. Part of Xiyan Li's Ph.D. dissertation 2006).

Phylogenetic analyses revealed a family of 4 ECAs in Arabidopsis, though their distinct functions were unknown. ECA3 differed from other ECAs, indicating that its function might be unique. First, ECA3 gene has 33 exons instead of 5-8 exons, and secondly, the protein sequence shared higher similarity with animal SERCA than with the other AtECAs. The expression levels of ECA3 in plants deduced by promoter::Gus analyses, though weak, was seen in the vasculature of vegetative and floral tissues, and in pollen. In a yeast mutant defective in its endogenous Ca pumps, expression of ECA3, restored yeast tolerance to Ca-depleted medium or to toxic levels of Mn (1 mM) (Fig. 1. Li et al. 2008 in press). A functional GFP-tagged ECA3 was localized to intracellular membranes of yeast, thus like ECA1, ECA3 was a Ca/Mn pump localized to the endomembrane.

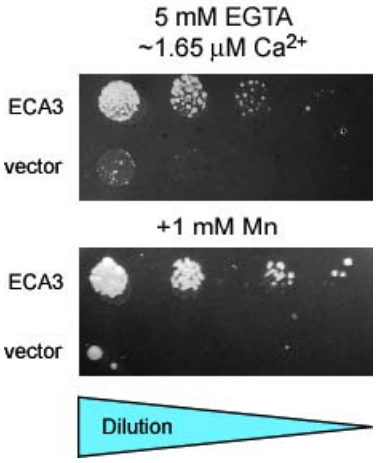

Fig. 1. **ECA3 restored K616 yeast growth on Ca-depleted medium** (top, ~1 µM Ca) and **on Mn-supplemented medium** (bottom). K616 (pmr1, pmc1, cnb1) was transformed with either vector alone or pYESDEST-ECA3. Transformants were normalized to OD_{600} of 1.0 and then

However, the inhibitor-sensitivity of ECA3 differed from ECA1. Thapsigargin (500 nM) as well as cyclopiazonic acid (200 nM) reduced growth ~20-30% in yeast expressing ECA3 on Cadepleted medium. This is the first evidence for thapsigargin-inhibition of a plant Ca pump at the molecular level. In vitro pollen tube growth was also impaired by nanoMolar levels of thapsigargin and of cyclopiazonic acid (Fig. 2), two specific inhibitors of animal SERCA, suggesting a role of ECA3 in pollen tube growth.

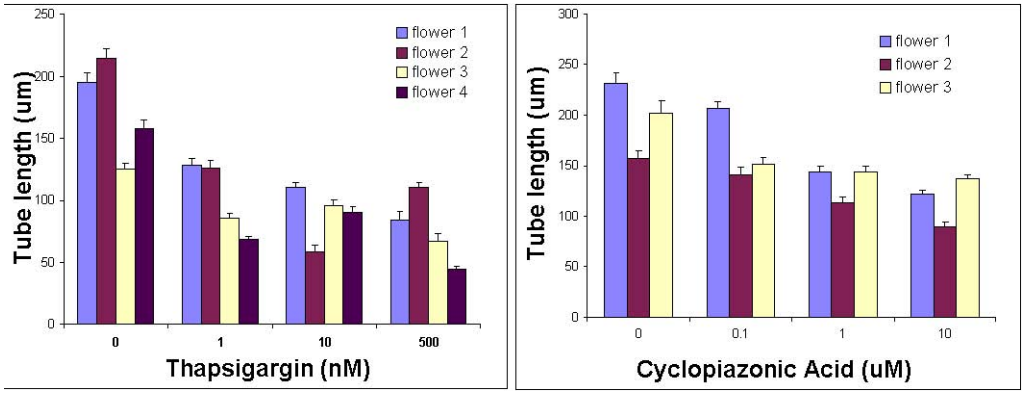

Fig. 2. **Thapsigargin and cyclopiazonic acid (CPA) decreased pollen tube growth in vitro**. Pollen from each of 4 flowers was tested at all conc.

Consistent with this idea, three independent alleles of T-DNA insertional mutants of eca3 displayed 33-48% reduced pollen tube growth in vitro. The inhibition of tube growth from eca3 mutants was particularly prominent at 10 mM Ca relative to 1 mM (Fig. 3). The defect in pollen tube growth reduced seed set by 13% of eca3 mutants. These results demonstrate for the first time that a Ca/Mn pump on endomembranes supports pollen tube elongation. Although the ER-localized ECA1 is also highly expressed in pollen tubes, tube growth of eca1 mutants was not significantly impaired probably due to the activity of a closely related ECA4 protein. Together, the results demonstrate that ECA3 supports pollen tube elongation and male fertility in a manner distinct from other ECAs.

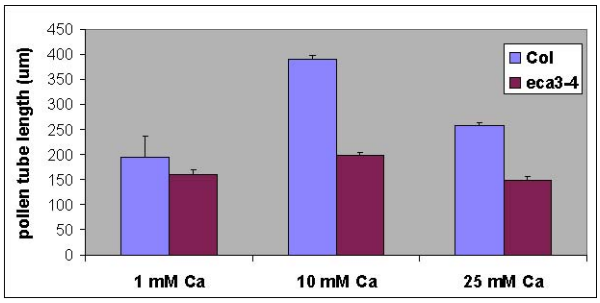

Fig. 3. **Pollen tube growth of eca3-4 mutant is significantly reduced**. Pollen from wild-type (Col) and mutant eca3-4 were germinated for 6 h at 26 C. Results are mean of 150-500 pollen tubes (error bar is S.E.). Data representative of 3 experiments.

iii) **An endomembrane Ca/Mn pump affects root growth through secretory activity** (Xiyan Li et al. 2008. Plant Physiol. In press)

A novel Ca/Mn pump AtECA3 on an endomembrane was shown to support pollen tube elongation and enhance male fertility; however the subcellular location was unclear and the molecular basis of its effect on growth was obscure. We have localized both GFP-ECA3 and ECA3-GFP to punctate structures of 1-2 um initially resembled the pattern of Golgi. Control GFP alone or GFP-tagged markers of vacuole (GFP-TIP), plasma membrane (CPK9), peroxisome (APX-GFP), or ER (GFP-HDEL) showed different fluorescent patterns. In co-transfection

experiments, ECA3 colocalized in part with Ara7, suggesting ECA3 is localized to PVC or post-Golgi compartments.

Three independent alleles of eca3 mutants showed a reduction in primary root growth at 3 mM Ca^{2+} ranging from 20-33% (Li et al. 2008). Shorter root length in mutants relative to wild type roots were also observed when external Ca was limiting (0.01 mM) as well as at high (20 mM) Ca, although the decrease in primary root length of mutant was greatest when wild-type roots showed maximum growth at 3 mM [Ca^{2+}]. These results indicate that maximal root growth depends on an adequate supply of external Ca, and that Ca transport into Golgi lumen by AtECA3 significantly influences the extent of root growth. Furthermore, when Ca is suboptimal or in excess, there is hardly any root growth in 2 out of 3 alleles of eca3 mutants. Together, these results demonstrate that loading Ca into a post-Golgi compartment by ECA3 serves a critical role in root elongation.

Mn, an essential micronutrient, stimulated primary root growth of wild-type seedlings at 3-50 µM; although higher levels Mn (0.1 to 2 mM) retarded growth. Intriguingly, root length of eca3 mutant (14 mm) was indistinguishable from the wild-type at 3 µM Mn ; yet at 50 µM Mn mutant root length was reduced to 5 mm compared to wild-type root length of 14 mm, indicating that mutants were unable to utilize this nutrient for growth as seen in Wt plants. The compromised root growth in mutant plants was accompanied by reduced leaf expansion and rosette size. The results would suggest that inability to load Mn into the post-Golgi compartment by ECA3 has handicapped the growth machinery. These results suggest that a certain amount of Mn and Ca inside post-Golgi compartment is essential for optimal growth.

iv) Deregulated secretion in eca3 mutants

To test whether eca3 mutants had defects in functions associate with post-Golgi compartments, we asked if secretory activity was compromised. The major roles of Golgi in plants are in the biosynthesis of pectins and hemicellulose as well as in the processing, sorting of soluble and membrane proteins, and secretion of wall proteins. Plants secrete peroxidases which function in cross-linking cell wall components and in defense. Apoplastic wall fluid was extracted from hydroponically grown plants. Peroxidase activity measured by guaiacol oxidation with hydrogen peroxide was increased in eca3-4 mutants 60% relative to wild-type (Li et al. 2008). The total amount of proteins secreted was also increased by over 60% per gm fw of roots, indicating that secretion of proteins is deregulated in eca3 mutants.

These results demonstrate that ECA3 Ca^{++}/Mn^{++} pump plays a critical role in the complex functions of post-Golgi endosomes including the secretion of wall proteins. Possibly, eca3 mutants have defects in the synthesis and processing of wall pectins and hemicelluloses. Thus eca3 mutants are unable to sustain growth rates of wild-type roots. Similar alterations in post-Golgi compartment functions could account for the reduction in pollen tube elongation (Fig. 3).

v) **Integrating transport with plant life: the male gametophyte** A functional genomic view of all transporters in plants

Kevin W. Bock, David Honys, John M. Ward, Senthilkumar Padmanaban, Eric P. Nawrocki, Kendal D. Hirschi, David Twell, and Heven Sze (2006). Integrating Membrane Transport with Male Gametophyte Development and Function through Transcriptomics. Plant Physiology 140: 1151-1168

As a first step to integrate the impact of transport processes on plant life, we were attracted to the male gametophyte, which represents a relatively simple model for systems biology. Here 'systems biology' is defined as the study of an organism, like the haploid male gametophyte, through its life-time using an integrative approach. For decades, ion dynamics (e.g. Ca^{++}, H^{+}, K^{+}) are known to influence pollen tube growth and guidance, although the molecular bases of the transporters mediating these ion fluxes are not known. One approach to identify transporters important for pollen tube elongation and thus for fertility is to highlight all the transporters expressed there. The male gametophyte has only two cell types, of which the vegetative cell is the most prominent in the mature grain and in a germinated pollen tube. To integrate transport with pollen development and function, a genome-wide analysis of transporter genes expressed in the male gametophyte at four developmental stages was conducted. Using ATH1 whole-genome microarray, Honys & Twell (2004) showed that ~60% of the genes are expressed in pollen. About 1269 genes encoding classified transporters were collected from the Arabidopsis thaliana genome. Of 757 transporter genes expressed in pollen, 16% or 124 genes, including AHA6, CNGC18, TIP1.3 and CHX08, are specifically or preferentially expressed relative to sporophytic tissues. Some genes are highly expressed in microspores and bicellular pollen (COPT3, STP2, OPT9); while others are activated only in tricellular or mature pollen (STP11, LHT7).

Analyses of entire gene families showed that a subset of genes, including those expressed in sporophytic tissues, were developmentally-regulated during pollen maturation. Early and late expression patterns revealed by transcriptome analysis are supported by promoter::GUS analyses of CHX genes and by other methods. Recent genetic studies based on a few transporters, including plasma membrane H^{+} pump AHA3, Ca^{2+} pump ACA9, and K^{+} channel SPIK, further support the expression patterns and the inferred functions revealed by our analyses (Sze et al. 2006 in "The Pollen Tube"). Early genes are generally involved in pollen development; while late genes participate in post-pollination events, like tube growth. Thus, these findings provide the groundwork to streamline functional studies of many specific transporter genes, and an opportunity to discover the functions of unknown proteins, including putative membrane receptors. Transport of ions and metabolites is intimately connected with signal transduction, cell wall metabolism, cytoskeleton rearrangement, and vesicle trafficking. Thus this type of analysis coupled with studies to determine transport activity, protein spatial distribution and interacting partners, will provide insights towards understanding the systems biology of the male gametophyte.

This study is a significant contribution to the scientific community, as we now have a nearly complete list of transporters in Arabidopsis that are annotated and classified according to their predicted function or phylogenetic relationship. Using the GO term for transport brings up only half of what we identified in random order. The working 'master' list of transporters we have generated in Bock et al. (2006) is particularly useful for those searching for expression of any transporter from many whole-genome transcriptome now available. The results reveal insights and infer functions, and thus are critical for generating working ideas to test many uncharacterized plant transporters in other plant cell types. For example, we have identified specific and key transporters expressed in guard cells (Kwak J, personal Comm.), mesophyll, and different root cell-types (Benfey P). Moreover, the list of Arabidopsis transporters has been used to identify in bulk all homologous transporters in rice (unpublished with Blake Meyer). (using http://mpss.udel.edu/).

Summary, Significance & Future Plans

Our studies of Ca pumps are demonstrating that imbalance in cytosolic [Ca] levels as well as lumenal [Ca] are important for plant growth and for the ability to tolerate environmental stresses. For example, *eca1* mutants defective in an ER-bound Ca pump, ECA1, showed reduced growth on medium with limiting Ca (0.2 mM); the results suggest that active loading of Ca into ER lumen is needed to promote growth (Wu et al. 2002). *eca3* mutants showed severe growth retardation of roots and pollen tubes on medium containing mM Ca (Li e tal 2008 in press). Since Ca uptake into cells is not impaired in eca1 or eca3 mutants (Wu et al. 2002), loading Ca into specific endomembrane compartment(s) must be limiting growth perhaps via perturbation of the secretory process (Li et al. 2008). We also show that the role of ECA3 is distinct from that of the more abundant ER-bound ECA1. ECA3 supports Ca-stimulated root growth and the detoxification of high Mn, possibly through activities mediated by post-Golgi compartments that coordinate membrane traffic and sorting of materials to the vacuole and the cell wall. Thus the homeostasis of ions in the lumen of ER, endosomes and possibly other compartments/vesicles of the secretory system are critical for promoting growth.

In addition to Ca, pH is also critical for signaling, though the molecular basis of this is less clear. We recently showed that mutants of a guard cell-specific cation/H+ exchanger failed to form a fully-open stomatal pore (Padmanaban et al. 2007), suggesting that regulating intracellular pH by endosomes is an important part of guard cell signaling. A long-range goal is to understand how plants regulate intracellular [Ca] and pH both of which play critical roles in modulating secretory activities. Cell wall synthesis and protein processing, sorting and secretion depend on the orchestrated coordination of many intracellular compartments/vesicles that make up the secretory system. The common cell biology question arising from our work is: how do plant cells mediate exocytosis and endocytosis in a spatially- and temporally-regulated manner. Our working hypothesis: is that the process depends in part on regulation of both Ca and pH dynamics. We are currently working to understand the roles of transporters predicted to modulate pH.

III. Budget:

There were no unexpended funds at the end of the grant period.

IV. Publications and presentations at meetings (supported in whole or in part by DOE)

(* submitted online 4 Plant Physiol papers Report # DOE/ER/20200-1 to DOE/ER/20200-4 http://www.osti.gov/elink-2413)

*Wu Z, F Liang, B Hong, JC Young, MR Sussman, JF Harper and H Sze (2002). An ER-bound Ca^{2+}/Mn^{2+} pump, AtECA1, supports plant growth and confers tolerance to Mn^{2+} stress. Plant Physiol. 130:128-37 (Report # DOE/ER/20200-1)

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

*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

*Li X, S Chanroj, Z Wu, SM. Romanowsky, JF. Harper, H Sze. (2008) A Distinct Endosomal Ca^{2+}/Mn^{2+} Pump Affects Root Growth through the Secretory Process. (in press Plant Physiol. June 08.

Li X, Rowanowsky S, Harper JF, Cheung A, Sze H. A SERCA-like Ca pump supports pollen tip growth and promotes male fertility. (ms in revision)

Reviews

Sze H, Liang F, Hwang I, Curran A, Harper JF (2000) Diversity and Regulation of Plant Ca^{2+} Pumps: Insights from expression in yeast. ***Annu Rev Plant Physiol Plant Mol Biol*** 51: 433-462

Sze H, Frietsch S, Li X, Bock KW, Harper JF (2006) Genomic and Molecular Analyses of Transporters in the Male Gametophyte. In 'The Pollen Tube' Ed. R. Malho. ***Plant Cell Monograph*** 3, 71 Springer-Verlag.

Meeting Presentation (partial list)

Li Xiyan, JF Harper, S Romanowsky, H Sze 2004. Calcium ATPase AtECA3 shows tissue-specific expression and supports root and pollen tube growth. Annual Plant Biology Meeting (July 24-28, 2004, Orlando, FL). Selected for oral presentation**.**

Bock KW, Honys D, Ward JM, Padmanaban S, Nawrocki, Hirschi KD, Twell D, H. Sze (2006) Integrating membrane transport with male gametophyte development and function through transcriptomics. 17th International Conference on Arabidopsis Research, Madison

Bock KW et al (2006) as above. Integrating membrane transport with male gametophyte development and function through transcriptomics. Plant Biology 2006. Poster Boston, MA. Aug. 3-9, 2006

Li X, Romanowsky S, Harper JF, Cheung A, Sze H (2007) An endosomal Ca/Mn pump supports growth of pollen tube and roots through functions of the secretory system. 14th International Workshop Plant Membrane Biology. Valencia, Spain.