UC Berkeley
UC Berkeley Electronic Theses and Dissertations

Title
Calcium Signaling and Protein Phosphoregulation in Plant Environmental Perception and Responses

Permalink
https://escholarship.org/uc/item/4dk3q10r

Author
Kleist, Thomas James

Publication Date
2015

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

Calcium Signaling and Protein Phosphoregulation in Plant Environmental Perception and Responses

By

Thomas James Kleist

A dissertation submitted in partial satisfaction of the requirements for the degree

of

Doctor of Philosophy

in

Plant Biology in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Sheng Luan, Co-chair
Doctor Peggy G. Lemaux, Co-chair
Professor Bob B. Buchanan
Professor Robert S. Zucker

Summer 2015

Abstract

Calcium Signaling and Protein Phosphoregulation in
Plant Environmental Perception and Responses

by

Thomas James Kleist

Doctor of Philosophy in Plant Biology

University of California, Berkeley

Professor Sheng Luan, Co-chair

Doctor Peggy G. Lemaux, Co-chair

Living organisms continuously monitor, interpret, and respond to their environments. They do so through an assortment of mechanisms, but the focus of this doctoral dissertation is on two strikingly prevalent mechanisms: calcium signaling and protein phosphoregulation. These two processes are linked through the activities of calcium-regulated protein kinases and phosphatases. This dissertation focuses on calcium signaling processes in plants and the role that calcium-decoding protein kinases, the elusive upstream calcium signal-coding complexes, and the downstream target proteins play in plant environmental responses. The described research builds upon recent advances in the field of plant genomics, merging bioinformatic methods with experimental approaches. Notably, much of the research utilizes genomic and transcriptomic data from an emerging model plant species, the moss *Physcomitrella patens*. Because all land plants are evolutionarily related, insights from mosses – an early-diverging plant lineage – are useful to classify proteins and assess functional conservation or divergence. In addition to synthesizing relevant published research, this dissertation describes the phylogenomic classification of a family of plant-specific calcium sensors known as calcineurin B-like proteins (CBLs) and their associated protein kinases (CIPKs). Plants feature several families of protein kinases that share a similar overall architecture: an N-terminal serine/threonine kinase domain coupled to a C-terminal autoinhibitory region. Some of these protein kinase families are either directly or indirectly regulated by calcium, the latter exemplified by CIPKs. Calcium-dependent protein kinases (CDPKs) are directly regulated by calcium through calcium-binding EF-hand domains in the autoinhibitory region. Both CIPKs and CDPKs constitute multi-gene families in all sequenced land plant genomes. In contrast, calcium/calmodulin-dependent protein kinases (CCaMKs) are found as single-

copy genes in most sequenced plant genomes and have been evolutionarily lost in certain plant lineages, including the preeminent model plant Arabidopsis. The accepted rationale for the inferred independent losses of *CCaMK* loci has been that CCaMKs fulfill obligate functions in plant-microbe symbioses that are incompatible with some plant lineages due to relatively recent evolutionary events. During the course of characterizing CIPKs, the unexpected observation that *Physcomitrella* – which is incompetent for canonical plant-microbe symbioses – contains two loci encoding CCaMKs prompted an investigation into CCaMK function in mosses. Through implementation and refinement of a gain-of-function approach in *Physcomitrella*, this doctoral research demonstrated that activation of a *Physcomitrella* CCaMK (CCaMK1) was associated with formation of brood cells, a type of stress-induced asexual propagule formed by mosses. In legumes, CCaMK regulates CYCLOPS, a novel type of transcription factor, by phosphorylating it at two sites in its autoinhibitory region. *Physcomitrella* contains a single CYCLOPS homolog that interacts with CCaMK1 and shows strong sequence conservation at the two key phosphosites identified by prior work in legumes. Expression of a modified form of *Physcomitrella* CYCLOPS containing two putative phosphomimetic substitutions at these sites also likewise elicited brood cell formation. These observations suggest that *Physcomitrella* CCaMK and CYCLOPS homologs operate in a similar manner to the legume CCaMK-CYCLOPS module; yet in *Physcomitrella*, the module tends to a disparate function. This dissertation further describes efforts to identify ion channels that function in calcium signal-coding processes. These efforts contributed to the identification of a novel family of putative cation channels through a combination of heterologous expression, electrophysiological techniques, and bioinformatic approaches. This doctoral dissertation encompasses a broad range of interconnected processes involved in calcium signal generation and interpretation in biology, and the field stands to address many unanswered questions detailed here. To that end, prospective ideas for further investigation are provided in the concluding remarks.

Table of Contents

Chapter 1: Introduction.

Land plants are an integral component of our ecosphere and contribute a large share of the macroscopic biological diversity found on earth today. Land plants have populated the earth roughly 400 - 500 million years (Mishler & Churchill, 1985; Kenrick & Crane, 1997). In the last roughly 12,000 years, humans have co-opted certain land plants for agricultural food production, thereby establishing the basis for modern civilization (Diamond, 2002). Angiosperms, or flowering plants, have been the source of germplasm for every major food crop. Because of their agricultural significance and vast extant diversity, angiosperms have also been the most intensively studied groups of land plants. As a reflection of this fact, all but two of the more than three dozen publicly available complete plant genome sequences are from angiosperm species at the present time. Angiosperms, despite their unparalleled importance, appeared rather recently in evolutionary time compared to other lineages of land plants, likely within the last 175 million years, (Jiao *et al*., 2011)

The earliest land plants evolved from an algal-like progenitor some 450 million years ago (Mishler & Churchill, 1985; Kenrick & Crane, 1997). Today, we recognize three lineages that diverged very early in the evolution of land plants, and these lineages — liverworts, hornwort, and mosses — are collectively referred to as bryophytes (Figure 1.1). In contrast to other extant plant lineages (i.e. vascular plants or tracheophytes), bryophytes have a gametophyte-dominant life cycle. Unlike tracheophytes, the vast majority of bryophytes are poikilohydric, and all characterized bryophytes require available water in their environment to undergo fertilization. Bryophytes exhibit several traits, including poikilohydry, that are reminiscent of inferred ancestral land plant traits (Mishler & Churchill, 1985). Although they have evolved independently for an estimated 350 - 450 million years, extant bryophyte lineages can provide valuable insights into early land plant evolution and gene function.

Because of the labile nature of terrestrial habitats in comparison to aquatic environments, early land plants must have had mechanisms in place to sense their environment and effectively respond to ensure survival, and these mechanisms can be expected to be evolutionarily retained among most extant land plant lineages. Consistent with this idea, mosses and other bryophytes have many similarities with angiosperms at the molecular level. Most canonical phytohormone signaling pathways are present in bryophytes, and many protein families are conserved across land plants and can be found in bryophytes (Rensing *et al.*, 2008). Research on bryophytes can inform our understanding of the evolution and physiology of all land plants. Because many gene or protein families show rapid radiation among angiosperms, bryophyte homologs can be used to anchor subfamilies among related protein sequences and help to identify core, conserved functions, and the genomes of early-diverging land plants may be expected to encode the essential components for land plant environmental sensation. In particular, interest in the molecular basis for environmental perception and responses in plants has been the underlying motivation for this dissertation. This dissertation is focused on the contribution of two prevalent and commonly interlinked cellular processes: calcium signaling and protein phosphoregulation. Transient rises in

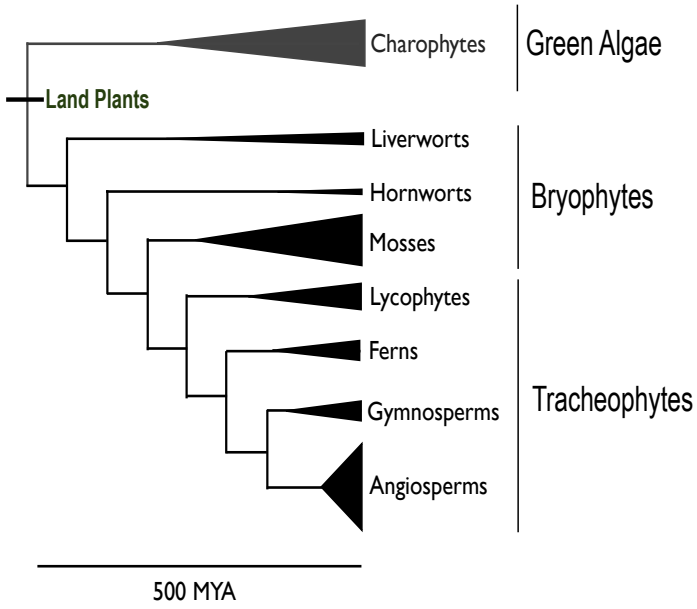


Figure 1.1: Evolutionary relationships among land plants and green algae. Cladogram summarizes inferred relationships among extant lineages of land plants and green algae (for further background, see Mishler & Churchill, 1985; Kenrick & Crane, 1997; and Jiao *et al*., 2011). Scale bar indicates approximately 500 million years ago (MYA). Wedges depict orthologous groups of organisms, widths are proportional to extant diversity.

nucleocytoplasmic calcium concentration, or calcium signals, are among the most rapidly occurring responses of plant cells to various environmental stimuli (Knight *et al.*, 1991; Knight *et al*., 1992; Ehrhardt *et al*., 1996}. Plant genomes encode a large variety of proteins endowed with calcium sensor activity, and a number of genes encoding calcium sensors have been linked to plant environmental responses. One way in which calcium sensors transmit their signals is by modifying the activity of enzymes, which can be located in the same or separate molecules. Among plants, protein kinases are an important class of enzymes that are regulated by calcium signals (Luan *et al*., 2002; Hrabak *et al*., 2003; Harper *et al*., 2004}. In conjunction with protein phosphatases, protein kinases regulate target proteins in an easily reversible manner, making them suitable molecular components for relaying the status of the ever-changing environment.

The available wealth of genome sequence data from plants and other eukaryotes, particularly publication of the complete genome sequence of the moss *Physcomitrella patens* in 2008 (Rensing *et al*., 2008), was an important catalyst and key resource for the majority of the work described in this doctoral thesis. Chapter 2 of this dissertation provides an extensive introduction to calcium signaling processes and protein phosphoregulation in the context of plant membrane transport regulation and environmental responses. Chapter 3 presents a phylogenomic classification of a family of calcium sensors (calcineurin B-like proteins, CBLs) and family of protein kinases (CBL-interacting protein kinases, CIPKs) that they regulate. Chapter 4 describes procedures for genetic transformation of *Physcomitrella* that were developed and optimized during research for this dissertation. Chapter 5 describes the functional characterization of a calcium-regulated protein kinase and target transcription factor in *Physcomitrella* that govern stress-induced asexual propagule formation. Chapter 6 summarizes efforts to characterize a family of membrane transport proteins that show calcium-permeable ion channel activity, and Chapter 7 contains concluding remarks.

Sources Cited.

Diamond, J. (2002) Evolution, consequences, and future of plant and animal domestication. *Nature* 418, 700-707.

Ehrhardt D. W., Wais R., & Long S. R. (1996). Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. *Cell* 85, 673-681.

Harper J. F., Breton G., & Harmon A. (2004). Decoding Ca^{2+} signals through plant protein kinases. *Annual Review of Plant Biology* 55, 263-288.

Hrabak E. M., Chan C. W., Gribskov M., Harper J. F., Choi J. H., Halford N., ... & Harmon A. C. (2003). The Arabidopsis CDPK-SnRK superfamily of protein kinases. *Plant Physiology* 132, 666-680.

Jiao Y., Wickett N. J., Ayyampalayam S., Chanderbali A. S., Landherr L., Ralph P. E., Tomsho L. P., Hu Y., Liang H., & Soltis P. S. (2011). Ancestral polyploidy in seed plants and angiosperms. *Nature* 473, 97-100.

Kenrick P., & Crane P. R. (1997). The origin and early evolution of plants on land. *Nature* 389, 33-39.

Knight M. R., Campbell A. K., Smith S. M., & Trewavas A. J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352, 524-526.

Knight M. R., Smith S. M., & Trewavas A. J. (1992). Wind-induced plant motion immediately increases cytosolic calcium. *Proceedings of the National Academy of Sciences U.S.A.* 89, 4967-4971.

Luan S., Kudla J., Rodriguez-Concepcion M., Yalovsky S., & Gruissem W. (2002). Calmodulins and calcineurin B-like proteins calcium sensors for specific signal response coupling in plants. *The Plant Cell* 14, S389-S400.

Mishler B.D., & Churchill S.P. (1985) Transition to a land flora: phylogenetic relationships of the green algae and bryophytes. *Cladistics* 1, 305–328.

Rensing S. A., Lang D., Zimmer A. D., Terry A., Salamov A., Shapiro H., ... & Rushton P. J. (2008). The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* 319, 64-69.

Timme R.E. & Delwiche C.F. (2010) Uncovering the evolutionary origin of plant molecular processes: comparison of *Coleochaete* (Coleochaetales) and *Spirogyra* (Zygnematales) transcriptomes. *BMC Plant Biology* 10, 96.

Chapter 2: Review and synthesis of scholarly literature on calcium signaling, protein phosphoregulation, and membrane transport processes.

The following chapter (excluding portions of the preface) has been accepted for publication as a peer-reviewed perspectives article in the journal *Plant, Cell and Environment* and is reproduced here with permission.

Kleist, T.J., and Luan, S. (2015) Constant Change: Dynamic Regulation of Membrane Transport by Calcium Signaling Networks Keeps Plants in Tune with Their Environment. *Plant, Cell and Environment* (accepted for publication).

Contributions.

T.J. Kleist provided intellectual contributions, prepared figures, and primarily wrote the manuscript, with assistance and intellectual contributions from S. Luan.

Preface.

Despite substantial variation and irregularities in their environment, plants must conform to spatiotemporal demands on the molecular composition of their cytosol and resident organelles. Cell membranes are the major interface between organisms and their environment and the basis for controlling the contents and intracellular organization of the cell. Membrane transport proteins govern the flow of molecules across membranes, and their activities are closely monitored and regulated by cell signaling networks. By continuously adjusting their membrane transport activities, plants can mitigate or buffer the effects of environmental perturbations, but effective implementation of this strategy is reliant on precise coordination among transport systems that reside in distinct cell types and different membranes. The following chapter examines the role of calcium signaling networks in the coordinated modulation of membrane transport, placing a special emphasis on transport of potassium, the most abundant cation in most plant cells. Classical and recent studies have underscored the importance of calcium ions in plant environmental responses and the regulation of membrane transport. While reviewing recent advances in our understanding of the the coding and decoding of calcium signals in plants, the established and emerging roles of calcium signaling in the coordination of membrane transport among multiple subcellular locations and distinct transport systems in plants are highlighted, drawing several examples from the CBL-CIPK signaling network. By synthesizing classical studies and recent findings, this chapter is intended provide timely insights on the role of calcium signaling networks in the modulation of membrane transport and its importance in plant environmental responses.

Introduction: Membrane Transport Research in the 'Omics Era.

Membranes provide selectively permeable barriers that interface among the cytosol, its resident organelles, and the environment. They permit the cell to establish an internal environment that can be stably controlled and that is suitable for physiological processes. For example, ATP is a vital molecule inside cells, where it serves as the primary energy storage molecule, among myriad other functions. Protein-mediated hydrolysis of ATP is dependent on magnesium (Mg^{++}), which acts as a cofactor and complexes with cytosolic ATP under physiological conditions. Because of the demand for high levels of ATP in the cell, the concentration of Mg^{++} must be maintained at rather high concentrations inside the cell (e.g. 1-10 mM in the cytosol) as well, to accommodate Mg-ATP binding. Contrarily, the similar divalent cation calcium (Ca^{++}) is toxic at comparable levels in the cytosol, in part because it causes precipitation of ATP and other phosphates (Clapham, 2007). This simple example implies that cell transport systems must be able to effectively discriminate between these similar divalent ions in order to maintain physiological processes.

Large fractions of plant genomes are devoted to membrane transport; roughly 5% of genes in sequenced plant genomes are thought to encode proteins that directly contribute to membrane transport (Sze *et al.*, 2013), though the vast majority of these genes lack any experimentally inferred function or activity. Plants are endowed with a network of membrane transport proteins (MTPs) that specialize in the transport of ions, metabolites, hormones, and other small molecules. The plant MTP network is distributed among the plasma membrane and organelles, therefore it can control the distribution of molecules among plant tissues, particular cells, and subcellular compartments. Constant change is a hallmark of membrane transport at all scales, from the on-off pulsing of a single ion channel to remodeling of entire transport networks. Controlled changes in membrane transport systems enable plants to counter environmental perturbations and serve as a homeostatic mechanism. In order to balance requirements from the subcellular to the whole-plant level and reconcile these requirements with environmental conditions, plants need environmental sensing and signaling systems with regulatory ties to membrane transport networks.

Plants reside in diverse and variable environments but require a steady, suitable balance of nutrients to maintain physiological processes. Membrane transport acts as an interface between metabolic demands and environmental constraints, hence the process is subject to continuous regulation in response to environmental fluctuation. Plants sense their environment and communicate this information through signaling networks. Similar to membrane transport, the plant signaling network is immense. Nearly 4% of protein-coding genes in fully sequenced plant genomes are believed to encode protein kinases (e.g. see (Lehti-Shiu & Shiu, 2012)), and roughly another 0.5% of genes appear to encode protein phosphatases (e.g. see (Moorhead *et al.*, 2009; Singh *et al.*, 2010)). Plant signaling networks integrate cues from the environment, metabolic processes, and developmental programs and decide on appropriate courses of action. Plant signaling systems implement membrane transport extensively, and membrane transport itself represents a major regulatory target for signaling networks.

Here, we examine some of the most intensively studied plant membrane transport and signaling systems to date and highlight how these two networks interconnect to mediate plant environmental responses.

Transport as a Coordinated Whole-plant Process: the Potassium Transport Network in Action.

Potassium (K^+) is the most abundant cation in plant cells and a fundamental requirement for plant growth. Physiologically, potassium fulfills many roles in plant cells. It acts as an important osmoticum and, due to its mobility, can be transported to adjust turgor pressure and govern plant movement. Potassium is used to maintain electrical charge balance, and it is a cofactor used my many enzymes. It is required for essential processes such as protein translation, sugar transport, and photosynthesis. Because of its importance in photosynthesis, shoot tissues require relatively greater amounts of potassium than root tissues. Potassium uptake, however, takes place almost exclusively in roots, therefore long-distance transport across the plant is required. Moreover, vacuoles contain a greater proportion of total plant potassium content than the cytosol, ergo potassium transport is a complex process that involves coordinated

Table 2.1: Families of potassium transport proteins in plants. The inferred subcellular localization and transport activity are listed for each family, with further information and references provided in the main text. Known physiological functions of characterized family members are also provided and described further in the text. The number of members from each family in rice (*Oryza sativa japonica*) and *Arabidopsis thaliana* were reported previously (Gomez-Porras *et al.*, 2012). Arabidopsis SOS1 and its homologs are described in a later section of the main text. Although they are not thought to directly participate in potassium transport, they have also been referred to as NHXs (e.g. SOS1 has also been called NHX7) and are included here for disambiguation (see Brett *et al.*, 2005).

Family		Subcellular localization	Transport activity	Physiological function(s)	# Members	
Name	Description				Arabidopsis	Rice
Shaker-like	Voltage-dependent K^+ channel	Plasma membrane	K^+ channel (voltage-dependent)	Environmental K^+ uptake, stomatal aperture control	9	11
TPK	Two-pore K^+ channel	Tonoplast	K^+ channel (voltage-independent)	Vacuolar K^+ release	5	3
KUP/HAK/KT	K^+ uptake permease / high-affinity K^+ / K^+ transporter	Plasma membrane, endomembranes?	K^+/H^+ co-transport; K^+ uniport	Environmental K^+ uptake, K+ homeostasis, osmoregulation, cell expansion	13	26
CHX	Cation / H^+ exchanger	Plasma membrane, tonoplast, endomembranes	K^+/H^+ co-transport	Cation homeostasis; pH regulation	28	16
NHX	Na^+/H^+ exchanger	Tonoplast, endomembranes	Na^+/H^+, K^+/H^+ co-transport	K^+ homeostasis; Na^+ sequestration; pH regulation	6	6
SOS1/NhaP-like	Salt Overly Sensitive 1 / Na^+/H^+ antiporter	Plasma membrane	Na^+/H^+ co-transport	Na^+ extrusion	2	1
HKT	High-affinity K^+ transporter	Plasma membrane	Na^+/K^+ co-transport; Na^+ uniport; cation channel	Na^+/K^+ homeostasis	1	7

movement both across the whole plant and amongst subcellular compartments in particular cells (reviewed by Maathuis & Amtmann, 1999).

Plants contain several families of membrane transport proteins that are implicated in potassium homeostasis (see Table 2.1; reviewed by Wang & Wu, 2013). The plant voltage-gated K^+ or Shaker-like family of ion channels are plasma membrane (PM)-localized channels that participate in potassium transport throughout the plant, and various members of the family have been specially adapted for functions in distinct cell types. The Arabidopsis genome contains 9 genes encoding Shaker-like channel subunits (see Figure 2.1), and these assemble into potassium channels with specialized functions. Shaker-like channels harbor a centrally located pore and have been shown to assemble into homo- or heterotetrameric structures (Daram *et al.*, 1997; Dreyer *et al.*, 1997). Shaker-like subunits can be classified according to phylogenetic and electrophysiological parameters into four categories: inward-rectifying, outward-rectifying, weakly rectifying, and silent channel subunits (Gomez-Porras *et al.*, 2012).

The inward-rectifying Shaker-like channel subunit AKT1 (ARABIDOPSIS K^+ TRANSPORTER 1) is important for potassium uptake from the environment and is believed to function across a wide range of external K^+ concentrations. It may act as the predominant route of entry for potassium ions into the plant in root hair and root cortical cells under high external K^+ concentrations; however Arabidopsis *akt1* loss-of-function mutants only show a growth phenotype when grown in low-K^+ (e.g. $\leq$ 100 micromolar) media (Hirsch *et al.*, 1998). In part, this is due to compensation by HAK5, a member of the KUP/HAK/KT (K^+ UPTAKE PERMEASE / HIGH AFFINITY K^+ / K^+ TRANSPORTER) family of transporters. HAK5 is required for potassium uptake from the environment when the external K^+ concentration is exceedingly low, but its transport activity is susceptible to inhibition by ammonium (NH_4^+) ions (Gierth *et al.*, 2005; Rubio *et al.*, 2008; Pyo *et al.*, 2010). Hence, the concentration of NH_4^+ present in the growth media strongly influences the low-K^+ phenotype of *akt1* mutant plants (Hirsch *et al.*, 1998; Spalding *et al.*, 1999). Under potassium-replete conditions, *HAK5* is transcriptionally repressed. Potassium starvation strongly induces *HAK5* transcription, to the extent that it has been adopted as a common marker gene for low-potassium stress conditions. Interestingly, phosphate or nitrate starvation can also induce transcription of *HAK5*, but these treatments do not lead to root high-affinity potassium uptake, implying that HAK5 is likely subject to some form of post-transcriptional regulation (Rubio *et al.*, 2014). Although Arabidopsis *akt1 hak5* double mutants show severe sensitivity to low-K^+ stress, they are able to grow similarly to wild-type plants when supplied with millimolar levels of K^+ (Pyo *et al.*, 2010; Rubio *et al.*, 2010). This implies the existence of another yet-to-be-identified potassium uptake system, distinct from HAK5, that either compensates for the absence of AKT1 activity or acts as the predominant potassium uptake system in wild-type plants under high-potassium conditions (Rubio et al, 2010; Caballero *et al.*, 2012).

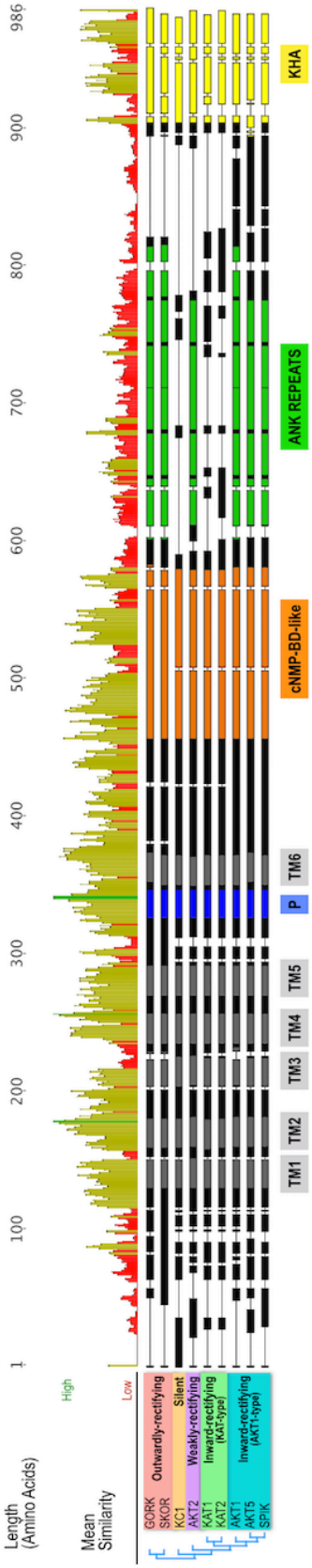


Figure 2.1: Phylogenetic relationships and domain architectures for Arabidopsis shaker-like potassium channels. Sequences were aligned using MAFFT, and the unedited multiple sequence alignment is shown with annotations. The N- and C-terminus of shaker-like channels are both intracellular. Each channel subunit contains six transmembrane helices (TM1-TM6, gray) and a pore-loop (P, blue) arranged in a (5)TM - P - TM configuration. The P-loop shows the highest degree of sequence similarity in the MSA. Each sequence contains a conserved region that, by homology, appears to be a cyclic mononucleotide phosphate-binding domain (cNMP-BD, orange), however its biological role has not been experimentally validated. Several Arabidopsis shaker-like channels contain ankyrin domain repeats (ANK, green), but these domains have been lost in the KAT1+KAT2 and KC1 clades. The conserved C-terminal domain (KHA, yellow) may be involved in channel tetramerization. Sequence similarity is plotted in the graphs above the multiple sequence alignment (MSA) with a sliding window = 3 amino acids.

In the *Xenopus* oocyte system commonly implemented for two-electrode voltage-clamp (TEVC) analysis, homomeric AKT1 channels are electrically silent when expressed alone, but they can be activated by coexpression with the calcium sensor CBL1 (CALCINEURIN B-LIKE 1) or CBL9 and its partner kinase CIPK23 (CBL-INTERACTING PROTEIN KINASE 23) in a phosphorylation-dependent manner, providing the first example of direct gating of a plant channel protein by phosphorylation (Li *et al.*, 2006; Xu *et al.*, 2006). Activation of AKT1 is abrogated by coexpression with AIP1 (AKT1-INTERACTING PROTEIN PHOSPHATASE 1) (Lee *et al.*, 2007), an A-Type protein phosphatase 2C (PP2CA), or its close homologs (Lan *et al.*, 2011). Hence, the CBL-CIPK-PP2CA regulatory module provides a putative on/off toggle mechanism to control AKT1 channel activity. Indeed, molecular genetic data corroborate this model, as *cbl1 cbl9* double mutants or *cipk23* single mutants show similar growth defects to *akt1* mutants under low-potassium conditions (Xu *et al.*, 2006; Cheong *et al.*, 2007), although genetic evidence for PP2CA involvement is heretofore lacking and requires further investigation.

The Arabidopsis shaker-like K^+ channel subunit KC1 (K^+ CHANNEL 1) was shown to be electrically silent when expressed in heterologous systems in its homomeric form, but KC1 can assemble with other inward-rectifying Shaker-like subunits to form active heteromeric channels. Heteromeric AKT1-KC1 channels can be activated by CBL1/9-CIPK23 in *Xenopus* oocytes, however heteromeric AKT1-KC1 channels show modified properties compared to AKT1 homomeric channels in *Xenopus* oocytes or plant cells (Reintanz *et al.*, 2002; Geiger *et al.*, 2009a; Jeanguenin *et al.*, 2011). Heteromerization of AKT1 with KC1 ostensibly leads to reduced leakage of potassium from the cytoplasm under very low external K^+ concentrations (Geiger *et al.*, 2009a), but the functional significance of AKT1-KC1 heteromerization may more directly relate to KC1-dependent regulation of heteromeric AKT1-KC1 channels by the SNARE protein SYP121 (SYNTAXIN-RELATED PROTEIN 121), a member of the syntaxin or Q-SNARE family (Honsbein *et al.*, 2009). KC1 reportedly interacts with SYP121 in split ubiquitin yeast two-hybrid (Y2H) assays, whereas AKT1 does not. Moreover, SYP121 promoted inward K^+ currents in oocytes coexpressing AKT1 and KC1 but not in ones lacking KC1 expression; and root protoplasts derived from *kc1*, *syp121*, or *akt1* mutant plants all showed similar defects in whole-cell inward K^+ currents (Honsbein *et al.*, 2009). Similar to the *akt1* growth phenotype, loss-of-function mutations in *KC1* or *SYP121* resulted in plant hypersensitivity to low-potassium conditions. Although the exact contribution of SYP121 to AKT1-KC1 channel regulation remains unclear, the authors have suggested that SYP121 is not merely involved in assembly or delivery of channels to the plasma membrane, noting that its coexpression with AKT1 and KC1 in oocytes (along with CBL1 and CIPK23 required for channel activation) was associated with changes in channel voltage-gating behavior (Honsbein *et al.*, 2009).

Trafficking of ion channels and other integral membrane proteins is a dynamic and ongoing process. SNARE proteins, such as SYP121, may participate in dynamic trafficking and help regulate the spatial distribution of Shaker-like channels among the plasma membrane and endosomes. In guard cells, potassium channels are subject to rapid regulation, where they assist in the opening and closure of the stomatal aperture

(Fischer, 1968; Humble & Hsiao, 1970). The stress phytohormone abscisic acid (ABA) acts upstream of cytosolic calcium signals and triggers ion efflux, along with concomitant loss of turgor, from guard cells (De Silva *et al.*, 1985; Schroeder & Hagiwara, 1989; McAinsh, 1990). Together with its paralog KAT2 (K^+ CHANNEL IN ARABIDOPSIS THALIANA 2), the inward-rectifying Shaker-like channel subunit KAT1 mediates potassium influx into guard cells and promotes stomatal opening (Pilot *et al.*, 2001; Szyroki *et al.*, 2001; Lebaudy *et al.*, 2008). Recombinant KAT1 tagged with fluorescent proteins (FPs) localizes to decipherable microdomains in the guard cell plasma membranes, and treatment with ABA specifically induced internalization of KAT1 (but not the PM H^+-ATPase PMA2) and its subsequent recycling to the plasma membrane (Sutter *et al.*, 2007). Hence, internalization of KAT1 has been interpreted as a negative regulatory mechanism that contributes to maintenance of stomatal closure. Interestingly, stomatal opening and remobilization of FP-tagged KAT1 channels to the plasma membrane were impaired in *syp121* mutants, hinting that SYP121 may, at least partially, confer stimulus-dependent mobility (Eisenach *et al.*, 2012). Although incorporation of the KC1 subunit has been proposed to serve as general regulatory mechanism for inward-rectifying Shaker-like channels (Jeanguenin *et al.*, 2011), it is unclear whether regulation of KAT1 by SYP121 is dependent on KC1. In metazoans, syntaxins functionally interact with synaptotagmins in a calcium-dependent manner; and calcium sensitivity is conferred by so-called C2 domains contained in the synaptotagmin moiety (Bennett *et al.*, 1992; Shao *et al.*, 1997). Hence, the dynamic trafficking and spatial distribution of ion channels provides another intriguing, hypothetical link between calcium signaling and membrane transport in plants.

Shaker-like channels exhibit diverse rectifying behavior; and despite the profound functional consequences, the structural basis for Shaker-like channel rectifying behavior appears to be quite flexible (Porée *et al.*, 2005; Riedelsberger *et al.*, 2010). The demonstration that single substitutions could switch the STELAR K^+ OUTWARD-RECTIFYING (SKOR) channel to a channel with inward-rectifying behavior in oocytes (Li *et al.*, 2008) underscores the structural flexibility of Shaker-like channel architecture and helps to explain their functional diversification during plant evolution. SKOR mediates potassium export into the xylem (Gaymard *et al.*, 1998); and consistent with its role in xylem loading, its activity is coupled to the available supply of potassium by a unique gating mechanism dependent on intracellular K^+ concentration (Liu *et al.*, 2006). Channels with disparate or opposite types of rectification commonly function together in the same cell in a coordinated manner. For example, the GUARD CELL OUTWARD-RECTIFYING K^+ (GORK) channel mediates cation efflux and stomatal closure (Ache *et al.*, 2000; Hosy *et al.*, 2003), hence it acts antagonistically to its homologs KAT1 and KAT2. Similarly, GORK mediates potassium efflux from roots under special circumstances (e.g. membrane depolarization) to maintain the membrane potential and ion homeostasis (Shabala *et al.*, 2006; Shabala & Cuin, 2008). Interestingly, the Shaker-like channel AKT2 exhibits two different gating modes: an inward-rectifying mode and a non-rectifying or “leak” mode (Dreyer *et al.*, 2001). AKT2 has been implicated in potassium transport in and out of the phloem, and disruption of AKT2 impairs sugar loading (Lacombe *et al.*, 2000; Deeken *et al.*, 2002; Gajdanowicz *et al.*,

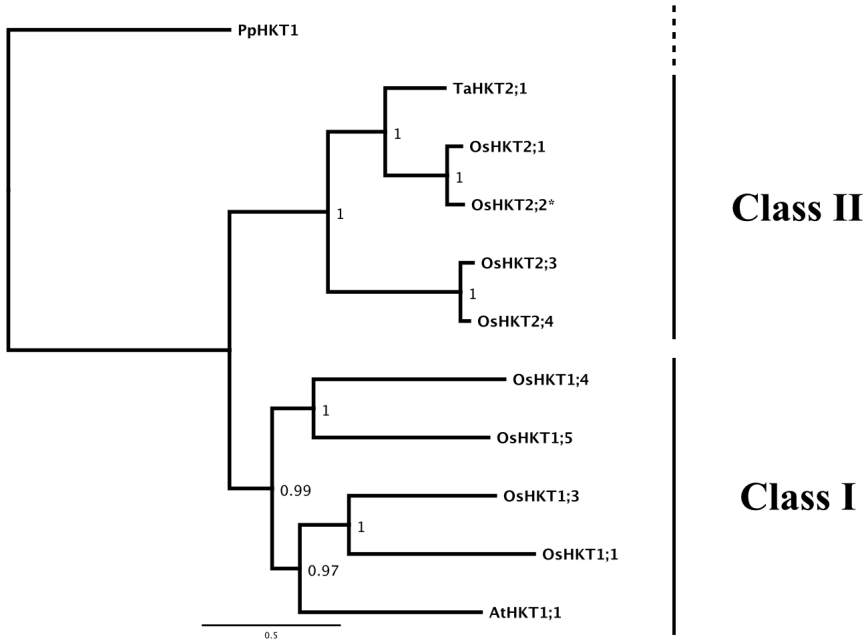
2011). The switch between AKT2 gating modes appears to depend on the phosphorylation status of AKT2 (Michard *et al.*, 2005), although the identity of the responsible kinases and the target residues have not been identified. The dual gating modes of AKT2 may relate to its physiological functions in the vasculature. In source tissues (e.g. roots), AKT2 mediates potassium entry into the phloem; however in sink or energy-limited tissues, AKT2 putatively supplements the activity of H^+-ATPases by allowing cation "leakage" from the phloem, thereby helping to maintain the negative potential across the plasma membrane required for sugar loading. The diverse and specialized activities of plant Shaker-like channels detailed here fit within a broader theme seen in other families of potassium transporters.

Whereas HAK5 has been implicated in the bulk supply of potassium to the plant, other members of the KUP/HAK/KT family appear to play uniquely specialized roles in determining cell shape and size. For instance, the semi-dominant *short hypocotyl 3-1* mutation was tracked to a single missense mutation in *KUP2*. The *shy3-1* mutation does not impair the potassium transport activity of KUP2; and loss-of-function *kup2* mutants lack a discernible phenotype, suggesting that not just the bulk supply of potassium, but the precise spatial control of potassium transport, is important for proper cell expansion (Elumalai *et al.*, 2002). Arabidopsis KUP4, which was initially identified as the *TINY ROOT HAIR 1* (*TRH1*) gene, is required for the expansion and normal development of root hairs (Rigas *et al.*, 2001). The phenotype of *trh1* loss-of-function mutants cannot be alleviated by elevated levels of potassium in the medium. Given that there are other MTPs capable of mediating potassium entry into the cell, the mutant phenotype is likely not caused by deficiencies in the bulk uptake of potassium but, rather, by tightly localized defects in potassium transport. Although elevated potassium levels cannot rescue the *trh1* phenotype, exogenous application of auxin reportedly can; and *trh1* mutants show aberrant auxin distribution and altered rates of auxin efflux (Vicente-Agullo *et al.*, 2004). Collectively, these observations point towards an important and sophisticated role for potassium transport in the regulation of cell shape and possible cross-talk or mechanistic links between potassium and auxin transport. Future work should focus on the hypothetical signaling mechanisms that regulate these transporters and finely tune cell expansion

Potassium is a finite resource for plants, and distribution of potassium among plant tissues is a coordinative decision made not only on the level of the entire plant, but also at the subcellular level. Intracellular potassium homeostasis is largely governed by sequestration and release from intracellular stores, most notably vacuoles. Using electrophysiological techniques, the vacuolar K^+-selective (VK) conductance was among the earliest channel activities observed using isolated plant vacuoles (Ward & Schroeder, 1994; Allen & Sanders, 1996). More recently, release of potassium from vacuoles has been attributed, at least in part, to TWO-PORE K^+ (TPK) channels (Gobert *et al.*, 2007; Dunkel *et al.*, 2008). TPKs do not show voltage dependency (Bihler *et al.*, 2005); however, they are gated by cytosolic calcium and pH (Czempinski *et al.*, 1997; Bihler *et al.*, 2005). In contrast to Shaker-like channels, TPK channels feature their own EF-hand pair and can be directly gated by calcium ions. TPKs have been linked to nutrient remobilization and potassium homeostasis (Gobert *et al.*, 2007). Theoretically,

vacuolar K^+ release should be critically important for processes such as seedling establishment and stomatal closure that are governed by calcium signaling networks, yet it is currently unclear whether TPKs participate in these processes. Unlike its other functions, potassium's role as a major osmoticum in vacuoles can be substituted, to some extent, with sodium. Potassium (K^+) and sodium (Na^+) ions are similar with respect to their chemical and physical properties, and various plant transport systems show overlap between potassium and sodium permeability. Aside from any role in osmotic regulation, sodium sequestration provides a means for detoxifying the cytosol when challenged with elevated levels of sodium. Vacuolar K^+/H^+ and Na^+/H^+ antiport activity were demonstrated using electrophysiological methods several years before the genetic identification of the responsible transporters (Blumwald & Poole, 1985; Garbarino & DuPont, 1988; Hassidim *et al*., 1990). Two families of transporters, Na^+/H^+ EXCHANGERs (NHXs) and CATION/H^+ EXCHANGERs (CHXs), have since emerged as major mediators of this process. The NHX (Venema et al., 2002; Venema et al., 2003; Liu *et al*., 2010; Barragán *et al*., 2012) and CHX (Cellier *et al*., 2004; Hall *et al*., 2006; Chanroj *et al*., 2011) families each contain members implicated in the transport of

Figure 2.2: Phylogenetic relationships and classification of HKTs from model plant species. HKTs have been divided into class I and class II HKTs. The characterized wheat root HKT (TaHKT2;1) mentioned in the main text is illustrated, but other wheat HKTs are excluded for simplicity. For all other species, the full complement of HKTs were included in the analysis. All rice HKTs are from the *japonica* variety except for OsHKT2;2 (*), which is a pseudogene in this variety and was substituted with the corresponding protein from the *indica* variety. Abbreviations used: *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Physcomitrella patens* (Pp), *Triticum aestivum* (Ta). The scale units are average substitutions per site, and the node labels show clade confidence metrics.



potassium, sodium, or both ions. Most characterized NHXs and CHXs have been reported to localize to vacuoles or endosomes; however, PM-localization has also been reported for CHX13 (Zhao *et al*., 2008). Because both the sodium and potassium transport activities of various NHXs and CHXs impact potassium homeostasis, they can be viewed as functionally interlinked processes. Furthermore, the intimate relationship between K^+ and Na^+ suggests that they must be jointly regulated by signaling networks.

Sodium toxicity is a common form of stress for plants in wild and agricultural settings, and plant lineages vary substantially in terms of genomic content and functional activities of their sodium and potassium transport system. The high-affinity K+ transporter (HKT) family is a prime example of this phenomenon. The first plant HKT (later designated TaHKT2;1) was identified by expression cloning using a cDNA library from wheat (*Triticum aestivum*) roots (Schachtman & Schroeder, 1994). Although initially proposed to function as a K^+/H^+ symporter for high-affinity potassium uptake, it was subsequently shown to function instead as a Na^+/K^+ symporter (Rubio *et al.*, 1995). Phylogenetically, plant HKTs can be divided into two classes (see Figure 2.2) based on three associated parameters: phylogeny, transport activity, and the amino acid identity at a key position in the selectivity filter of the first pore-loop. Class I HKTs, which contain a serine (Ser) residue in the first pore loop, have been reported to operate as transporters with a strong preference for Na^+ over K^+; whereas class II HKTs contain a glycine (Gly) residue at the same position and, like TaHKT2;1, may operate as Na^+-K^+ co-transporters. The transport activity of either class of HKT can be experimentally swapped by introducing a Ser-to-Gly substitution or vice-versa. Class II HKTs more closely resemble HKTs from non-plant organisms, and the single HKT from *Physcomitrella* contains a Gly at the aforementioned position in its selectivity filter thus resembles a class II HKT in this respect. Whereas grasses typically contain multiple HKTs distributed among class I and II, Arabidopsis contains only one HKT (AtHKT1;1) that belongs to class I. It has been convincingly shown to function in xylem parenchyma cells and retrieve sodium ions from the xylem stream to aid in detoxification (Davenport *et al*., 2007). Despite initially being viewed strictly as high-affinity transporters, studies on various rice HKTs have indicated that some HKTs operate as ion channels with permeability to monovalent and divalent cations, including calcium (Lan *et al*., 2010; Horie *et al*., 2011). Consistent with the observed channel-like activity of some class II HKTs, computational modeling suggests striking structural similarity among HKTs and prokaryotic KcsA channels (Durell & Guy, 1999). The genomic complexity of the plant potassium transport machinery detailed here and the interplay between competing potassium and sodium ions underscore plants' requirement for diverse MTPs that act in a specialized yet coordinated manner. Next, we examine the role of calcium signals in coordinating plant membrane transport and responding to environmental perturbations.

Plant Environmental Perception: Calcium Signal-coding Networks.

Plant environmental responses heavily entail modulation of their membrane transport system. Unsurprisingly given the anatomical complexity of plants, stress signaling and responses are specialized in a cell-type dependent manner. Coordination

of stress responses is achieved by extensive short-distance and long-distance communication. The signaling networks that plants use to communicate are highly interconnected and feature abundant feedforward and feedback loops; this network layout is thought to be a key principle in plants' ability to robustly respond to environmental perturbations. Hormones like auxins, cytokinins, and ABA can be transported long-distance in plants and help coordinate plant environmental responses by regulating diverse signaling pathways in a synchronized manner. Similarly to hormones, calcium signals serve to integrate environmental signals and coordinate plant responses; mounting evidence suggests that calcium "waves" can also act as long-distance signals in plants.

Although plant environmental responses have been well documented, including at the molecular level, the molecular basis of plant sensation itself is unknown. Membranes define the boundaries of cells and their organelles and are sources of potential energy due to the electrochemical gradient. Many MTPs, particularly those that show ion channel activity, are gated by physical parameters such as stretch, whether induced by physical contact, osmotic perturbations, or temperature-dependent changes in fluidity. Despite the fluid nature of lipid bilayers, cell membranes exhibit considerable structure, which is conferred by local lipid composition, scaffold proteins, cytoskeletal elements, and the cell wall (or extracellular matrix in animal cells). Proteins involved in membrane transport are capable of directly sensing their environment and responding by changes in transport activity. As crudely demonstrated by the usage of various ion species as ion channel blockers, small molecules can directly influence the activity of MTPs. This principle was leveraged to discover the molecular basis for thermal sensation in animals. Capsaicin, which invokes the sensation of heat, and menthol, which evokes the sensation of cold, achieve their characteristic sensory response by direct activation of transient receptor potential (TRP) ion channels that mediate temperature perception (Caterina *et al*., 1997; Peier *et al*., 2002). Other TRPs mediate sensory processes such as touch, osmotic responses, and vision (Clapham, 2003). Transmembrane channel-like proteins (TMCs) are another family of ion channels that have been firmly linked to metazoan sensory perception (Pan *et al*., 2013). Mutations in TMC1 and TMC2 in mice or humans are associated with deafness (Kurima *et al*., 2002); and, in the roundworm *Caenorhabditis elegans*, a TMC homolog appears to act as a sodium sensor (Chatzigeorgiou *et al*., 2013). As evidenced by these examples, MTPs can directly mediate environmental sensation and act at the earliest stages of responses to environmental stimuli.

The genetic identification of calcium-binding proteins required for certain stress responses and the demonstration that calcium-binding domains act as regulatory regions of stress-response proteins further implies that calcium signals, which are observed in response to a variety of stresses (Knight *et al*., 1991; Knight *et al*., 1997), play a causative role in plant stress responses. Nonetheless, few channels have been directly shown to mediate calcium signals in planta; and despite intensive efforts, putative sensory calcium channels have not yet been placed in the genetic context of known signaling pathways in plants, to our knowledge. Receptor-like kinases (RLKs) and other types of proteins may fulfill these functions instead or in parallel to calcium-

permeable channels; and, importantly, these scenarios are not mutually exclusive. RLKs commonly possess extracellular domains that, in some cases, mediate interaction with cell wall components, thereby providing a hypothetical tether between the membrane and the wall, which might conceivably endow sensory activity (Osakabe *et al*., 2013).

Calcium-permeable channels remain tantalizing candidates for stress sensors in plants because of the rapid changes in cytosolic calcium levels observed in response to environmental perturbations and stress-related phenotypes that have been traced to mutations in genes encoding putative calcium sensors. Plants contain a few families of proteins with clear homology to channels involved in calcium signal generation in animals. These have been widely suggested to participate in calcium signal generation and include cyclic nucleotide gated channels (CNGCs) (Leng *et al*., 1999; Finka *et al*., 2012), glutamate-like receptors (GLRs) (Lam *et al*., 1998; Mousavi *et al*., 2013), and vacuolar two-pore channels (TPCs) (Furuichi *et al*., 2001; Peiter *et al*., 2005). Nonetheless, the molecular identities of hypothetical sensory MTP complexes in plants remain unclear, largely owing to limited genetic evidence. Like plants, the yeast *Saccharomyces cerevisiae* rapidly responds to environmental perturbations with distinctive patterns of cytosolic calcium elevation. Whereas plants lack discernible TRP or voltage-gated calcium channel (VGCC) homologs, *Saccharomyces* contains one of each. The TRP homolog, named YEAST VACUOLAR CONDUCTANCE 1 (YVC1), mediates vacuolar calcium release in response to hyperosmotic stress, however yvc1 deletion strains do not cause any identified growth defect. The VGCC homolog, CCH1 (CALCIUM CHANNEL HOMOLOG 1), forms a calcium permeable channel with MID1 (MATING-INDUCED DEATH 1) and is generally agreed to constitute the pore-forming subunit of channel. Yeast *cch1*, *mid1*, or *cch1 mid1* deletion strains show identical phenotypes: cells die when exposed to mating pheromone alpha specifically in low-calcium media. This phenotype has been leveraged to screen plant genes that functionally complement yeast mid1 mutants and led to the identification of Arabidopsis *mid1*-COMPLEMENTING ACTIVITY (MCA) proteins, which have been proposed to act as stretch-activated calcium channels in plants (Nakagawa et al., 2007; Yamanaka et al., 2010). Sensory calcium channel activity may be traced to proteins with non-canonical features for ion channels. Annexins are unusual proteins that can associate with phospholipids or reside in the cytosol. Unlike canonical MTPs, they are not thought to follow the secretory pathway, and annexins have been considered as calcium sensors because their core domain mediates calcium binding and membrane interactions in a manner analogous to C2 domains (Gerke *et al*., 2005). Recently, however, Arabidopsis annexin 1 (ANN1) was shown to be required for hydroxyl radical- and hydrogen peroxide-induced calcium transients in planta, and calcium transport activity reminiscent of ion channels was demonstrated in artificial bilayers embedded with immunopurified protein (Laohavisit *et al*., 2012; Richards *et al*., 2014). Using a candidate gene approach, Arabidopsis calcium permeable stress-activated channel 1 (CSC1) was identified as the founding member of a eukaryotic-wide family of ion channels by screening for Arabidopsis genes whose expression mediated osmosensitive cytosolic calcium elevation in Chinese hamster ovary (CHO) cells and

Xenopus oocytes (Hou *et al.*, 2014). A close paralog of CSC1 was identified as OSCA1 (REDUCED HYPEROSMOLARITY-INDUCED CYTOSOLIC CALCIUM INCREASE 1) through forward genetic screens using Arabidopsis lines expressing the calcium indicator aequorin (Yuan *et al.*, 2014). Despite these efforts, none of the aforementioned candidates have been definitively shown to function as sensory channels in planta.

Identifying MTPs that fulfill hypothetical roles in environmental sensing has been a longstanding goal among plant biologists, but molecular genetic efforts have heretofore yielded few interpretable results. Parallel or interconnected signaling pathways could obfuscate standard loss-of-function genetic approaches due to the requirement for mutations in multiple pathways; and because parallel pathways may expectedly be comprised of non-homologous components, they may stymie computational approaches. An example of this biological principle is provided by the yeast high osmolarity glycerol (HOG) pathway. The *S. cerevisiae* HOG1 protein is a MAPK (MITOGEN ACTIVATED PROTEIN KINASE) that serves as a master regulator of the yeast osmotic stress response. HOG1 can be activated by two branches that feed into the HOG pathway: the SLN1 branch and the SHO1 branch. Yeast strains only show growth defects in response to hyperosmotic stress when both input branches of the HOG pathway are genetically disabled (O'Rourke & Herskowitz, 2002; Tatebayashi *et al.*, 2007). Hence, parallel inputs of environmental signals can impede functional genetic analysis and necessitate multiple loss-of-function mutations in non-homologous genes.

Recent advances in calcium imaging techniques in live plant cells have enabled unprecedented characterization of calcium signals in plants, thereby narrowing the gap in our knowledge of plant stress responses. Calcium ions are rapidly chelated in the cytoplasm, for example, by protein carboxylate groups, and sequestered or expelled by transporters; therefore cytosolic calcium signals are under tight spatiotemporal constraints, as demonstrated by simulation models and experimental validation (see review by Martins *et al.*, 2013). Calcium imaging experiments reveal, however, that calcium waves are transmitted not only across a cell, but across long distances in the plant (Mousavi *et al.*, 2013; Choi *et al.*, 2014). The mechanism of wave propagation is presumed to operate, at least in part, by calcium-induced calcium release, though the exact details are unclear. Calcium-gated MTPs that contain their own EF-hands, such as TPC1, have been implicated in the process (Choi *et al.*, 2014). Moreover, given known mechanisms of calcium wave propagation in metazoans, membrane depolarization is a potential driving mechanism for this phenomenon. Increasing evidence seems to point towards long-distance calcium waves as key sensory for environmental sensation in plants.

Signal Integration in Plant Environmental Responses: Calcium-decoding Networks.

Considerable progress has been made in recent years regarding the functions and mechanisms of calcium-decoding networks in plants, and it is clear that calcium signaling networks play a coordinative role in regulation of membrane transport. Major challenges moving forward include the genetic identification of MTPs that generate calcium signals and characterization of calcium signaling events and outcomes in response to concurrent stresses, as might be expected to occur in natural or agricultural settings.

Calcium sensor proteins typically bind calcium via either beta-sandwich structures known as C2 domains or helix-loop-helix structures known as EF-hands. Calcium binding to C2 domains acts like an electrostatic switch and modulates protein affinities for phospholipids and – consequently – membrane association, as well as protein-protein interactions (Shao *et al.*, 1997, Rodriguez *et al.*, 2014). In contrast, calcium binding to EF-hands promotes protein conformational changes. Calmodulins (CaMs), which are calcium sensors conserved across eukaryotes, have long been implicated in the regulation of MTPs such as CNGCs (Bethke & Jones, 1994; Schuurink *et al.*, 1998; Kohler *et al.*, 1999; Leng *et al.*, 1999) and H^+- or Ca^{++}-ATPases (Siegel & Hang, 1983; Bonza *et al.*, 1998; Harper *et al.*, 1998; Schuurink *et al.*, 1998; Chung *et al.*, 2000) through direct physical interactions and calcium-modulated conformational changes. Interactions between CaMs and MTPs can be transient or persistent. In some cases, CaMs might be defined as subunits of MTP complexes (Saimi & Kung, 2002). CaMs contain four EF-hand domains clustered into two pairs and a long hydrophobic region spanning the EF-hand pairs, yielding an overall structure that resembles a dumbbell. Calcium binding triggers CaM conformational changes that impact the activities of target proteins. In the case of MTPs, these conformational changes affect their transport activity and provide a mechanism to link cytosolic calcium status to membrane transport regulation.

Proteins involved directly or indirectly in membrane transport are ubiquitously regulated by post-translational modifications (PTMs). These may include familiar processes such as redox regulation, ubiquitination, or lipid modifications; however similar to soluble protein regulation, reversible protein phosphorylation is a particularly salient theme in MTP regulation. Many plant protein kinases associate with membranes and are regulated by calcium, linking membrane transport to cytosolic signaling networks. Calcium-dependent protein kinases (CDPKs) are a large family of protein kinases in plants that directly respond to elevated calcium concentrations via C-terminal EF-hands. Of the 34 CDPKs identified in Arabidopsis, 27 of them contain N-myristoylation motifs (Hrabak *et al.*, 2003), pointing towards their possible involvement in membrane-associated processes. Indeed, multiple CDPKs have long been linked to ion transport regulation (Li *et al.*, 1998; Hwang *et al.*, 2000). More recently, CDPKs have been shown to play important roles in linking ABA signaling to membrane transport in guard cells in order to control gas exchange and water loss (Mori *et al.*, 2006; Geiger *et al.*, 2009b; Brandt *et al.*, 2012; Scherzer *et al.*, 2012). Calcineurin B-like

proteins (CBLs) are a family of small (~200 amino acid), tetra-EF-hand calcium sensors in plants that bear homology to the B regulatory subunit of calcineurin, an important protein phosphatase in animals and yeast. Unlike CaMs, CBLs appear to specifically regulate the activities of one family of protein kinases, termed CBL-interacting protein kinases (CIPKs); and the CBL-CIPK network has emerged as a broad overseer of plant ion transport.

Network Connections: the CBL-CIPK Paradigm of Membrane Transport Regulation.

The CBL-CIPK network plays important roles in decoding cytosolic calcium signals and modulating the activities of plant MTPs. CBLs are small proteins (~200 amino acids) that share a simple domain architecture (see Figure 2.3). The N-terminus harbors a localization motif that mediates membrane interactions, and four calcium-binding EF-hands are distributed in two pairs. The first EF-hand of CBLs is distinctive in that it contains a 14-amino acid calcium-binding loop rather than the 12-amino acid calcium-binding loops found among canonical EF-hands, and structural data suggest that it has a unique mode of calcium binding (Nagae *et al.*, 2003; Sánchez-Barrena *et al.*, 2005). CIPKs contain an N-terminal Ser/Threonine (Thr) protein kinase domain and a C-terminal autoinhibitory domain. A hydrophobic cleft of the CBL binds to a conserved region known as the NAF or FISL motif (named according to conserved amino acids) located in the C-terminal autoinhibitory domain of CIPKs (Shi *et al.*, 1999; Kim *et al.*, 2000; Albrecht *et al.*, 2001; Guo *et al.*, 2001; Sánchez-Barrena *et al.*, 2007; Akaboshi *et al.*, 2008).

A recent study (Chaves-Sanjuan *et al.*, 2014) revealed the structural basis for CIPK autoinhibition by the region containing the NAF motif: this region acts as a pseudosubstrate and occupies the active site of the kinase, thereby blocking its activity. Coupled with prior biochemical evidence that CBL-CIPK physical interactions are calcium-dependent, this observation strongly implies that CBL-CIPK complex formation is dynamic in plant cells, as inactivation of the kinase and signal abrogation seemingly depends on dissociation from the CBL. As described for AIP1, PP2Cs can serve as negative regulators to counter activation of CBL-CIPK signaling pathways. CIPKs contain a region in their C-terminus known as the protein phosphatase interaction (PPI) motif that mediates physical interactions with CIPKs (Ohta *et al.*, 2003; Lee *et al.*, 2007; Lan *et al.*, 2011), however further investigation is needed because it has been shown that PP2Cs can still regulate and interact with a CIPK with the PPI motif experimentally deleted (Lan *et al.*, 2011). CBLs and PP2Cs also appear to directly interact physically and functionally with each other (Lan et al., 2011), therefore there are several promising avenues for the molecular genetic dissection of CBL-CIPK-PP2C functional interactions in plants. These approaches can be supplemented by computational and experimental studies of CBL-CIPK-PP2C structural dynamics. The functional and structural relationships of a few CBL-CIPK pairs are well established, and these examples provide a template for studies of other CBL-CIPK pairs. CBLs govern the localization and activation of CIPKs, and CIPKs confer the enzymatic activity and presumably contribute

to target specificity (Luan, 2009). Hence, present efforts have focused on identifying functional connections among CBLs and CIPKs, CBL-CIPK targets, and signaling components that crosstalk with the CBL-CIPK network.

CBLs have been classified according to their phylogenetic relationships and N-terminal localization motifs (Batistič *et al.*, 2008; Batistič & Kudla, 2009; Kleist *et al.*, 2014). The most thoroughly characterized CBLs contain the dual-lipid modification motif MGCXXS/T at their N-termini and are termed Type I CBLs. This motif triggers cleavage of the first position methionine, N-myristoylation of the Gly residue, and S-acylation of the subsequent cysteine (Cys) residue. Together, these modifications mediate associations with the plasma membrane. Although covalent attachment of the myristoyl group is irreversible, some neuronal calcium sensor (NCS) proteins feature a calcium-dependent myristoyl switch that exposes the myristoyl moiety when bound to calcium to facilitate membrane targeting but hides it in its hydrophobic cleft at resting calcium levels when the protein does not bind calcium (Zozulya & Stryer, 1992). It is unclear whether CBLs might exhibit a similar mechanism. Protein S-acylation is a reversible post-translational modification that is performed by protein S-acyltransferases and removed by S-acyl protein thioesterases. Whereas N-myristoylation is unique to Type I CBLs, computational evidence suggests that the Cys residue targeted for S-acylation in Type I CBLs is conserved among all identified CBLs (Kleist *et al.*, 2014), and molecular genetic evidence (e.g. see (Batistič *et al.*, 2008; Batistič *et al.*, 2012)) has demonstrated that this residue is functionally required by many, if not all, CBLs. It has become clear that the same CIPK can be targeted to distinct subcellular locations to fulfill multiple functions by partnering with different CBLs. CBL-CIPK complexes can therefore link distinct subcellular compartments through cytosolic calcium signaling processes and broadly coordinate membrane transport and ion homeostasis.

At least two other types of CBLs have been recognized: Type II CBLs, which harbor a Tonoplast Targeting Sequence (TTS) at their N-terminus, and Type III CBLs, which have a predicted N-terminal transmembrane helix. Type III CBLs likely function at the tonoplast and contribute to salinity tolerance (Kim *et al.*, 2007; Quan *et al.*, 2007; Tang *et al.*, 2014). Type II CBLs form a large clade that is conserved among early-diverging plants including bryophytes (Kleist *et al.*, 2014). CBL2 and its paralog CBL3 broadly regulate vacuolar membrane transport, as loss-of-function mutations lead to pleiotropic effects on ion homeostasis. This observation has been rationalized by demonstration that CBL2 and CBL3 functionally regulate the vacuolar ATPase (V-ATPase) complex (Tang *et al.*, 2012), thereby controlling trans-tonoplast transport of various ions that requires a pH gradient across tonoplast. In addition, our recent work (Tang *et al.*, 2015) shows that CBL2 and CBL3 also regulate transport processes that are independent of V-ATPase activity. For example, CBL2 and CBL3 appear to positively regulate a transporter or channel responsible for vacuolar sequestration of excess magnesium (Mg^{++}), leading to detoxification of the cytosol under high Mg^{++} stress (Tang *et al.*, 2015). This study also identified four CIPKs that appear to interact with CBL2 and CBL3 at the tonoplast and function in the same pathway for Mg^{++} tolerance, which underscores the interconnected or modular nature of the CBL-CIPK signaling network.

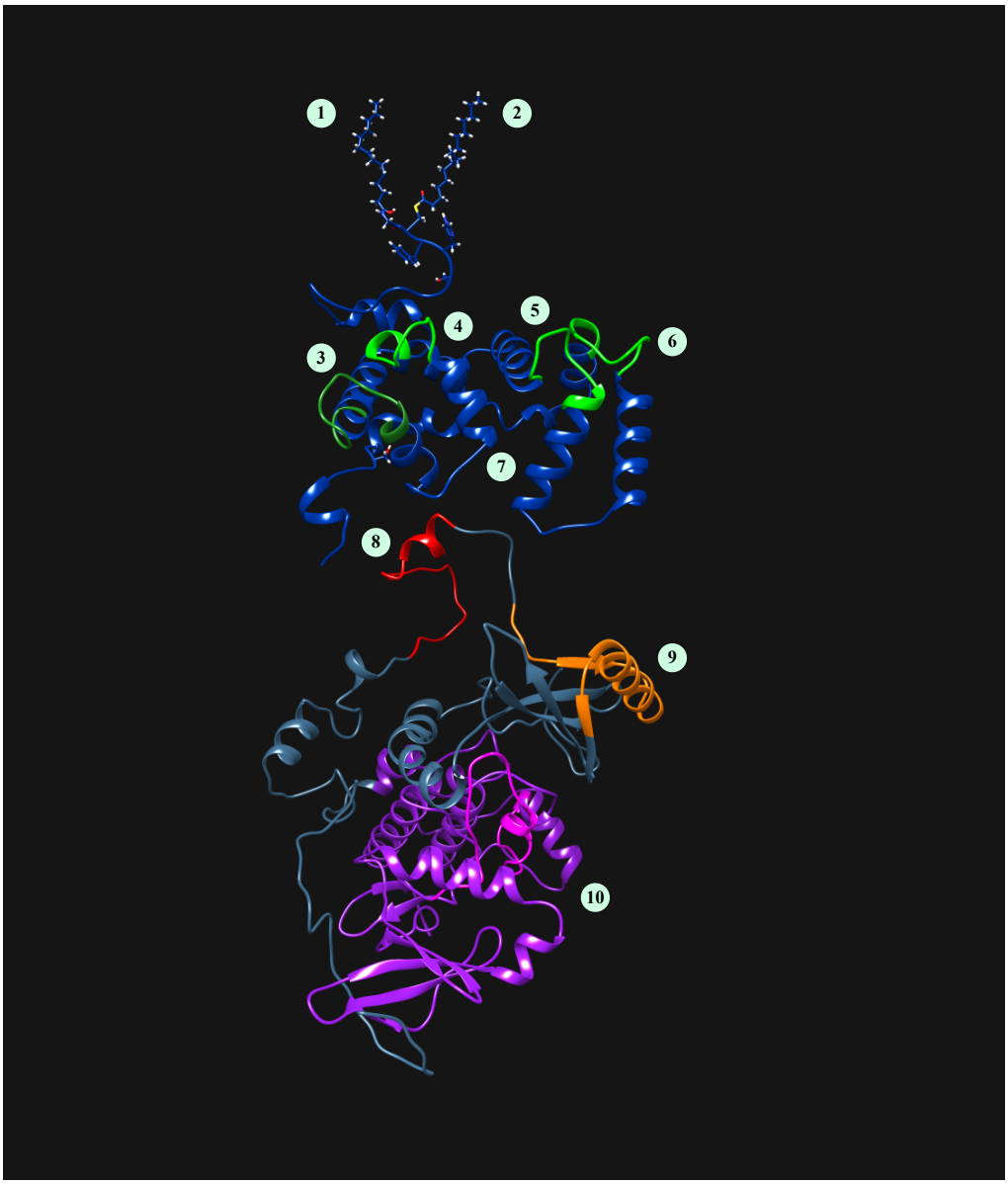


Figure 2.3: Annotated structural models showing key features of Arabidopsis CBL1 (top) and CIPK23 (bottom). Proteins are arranged in a hypothetical pre-complex state. (1) After cleavage of the N-terminal methionine, the subsequent glycine residue is N-myristoylated. (2) The adjacent cysteine residue is also lipid modified by covalent attachment of stearate or palmitate (pictured). (3-6) CBL1 contains four EF-hands (EF1-4, green) that form two pairs: EF1+EF2 and EF3+EF4. EF1 (3) has a distinctive 14-amino acid calcium-binding loop that binds calcium in an unusual manner. EF2-4 contain canonical 12-amino acid calcium-binding loops. (7) The CBL hydrophobic cleft mediates CIPK binding. (8) The NAF domain (red) confers CBL binding and occupies the CIPK active site in the CBL-unbound state. (9) The PPI domain contributes to interactions among CIPKs and PP2Cs. (10) The kinase domain (purple) of CIPK23 is similar to other serine/threonine protein kinases with the activation loop highlighted (pink).

Cytosolic signaling networks coordinate the transport of competing ions, for example potassium and sodium. In addition to the previously described CBL-CIPK regulation of potassium transport, the Arabidopsis salt overly sensitive (SOS) pathway provides another well-characterized example of CBL-CIPK function. SOS1 is a plasma membrane-localized Na^+/H^+ antiporter that is activated by CIPK24 (SOS2) in concert with CBL4 (SOS3) to promote salinity tolerance under high-sodium conditions (Qiu *et al.*, 2002; Quintero *et al.*, 2002). CIPK24 phosphorylates the C-terminus of SOS1 in a CBL4- and calcium-dependent manner and removes SOS1 autoinhibition (Quintero *et al.*, 2011). Activation of SOS1 leads to extrusion of sodium ions across the plasma membrane and a decrease in cytosolic sodium, which is toxic to most plants. Therefore, the CBL-CIPK pathways regulating SOS1 and AKT1 have distinct but complementary functions in potassium nutrition.

CBL-CIPK functions at the plasma membrane extend beyond potassium nutrition and sodium toxicity. For instance, CIPK23 has been shown to regulate the nitrate transporter CHL1 via phosphorylation (Ho *et al.*, 2009), providing an example wherein the same CIPK functions at the same subcellular location to coordinately regulate the transport of the distinct substrates of AKT1 and CHL1. The CBL-CIPK network was also shown to regulate H^+-ATPase activity in plants. RNAi lines with reduced CIPK11 expression or *cipk11* mutants showed increased tolerance to mildly alkaline (pH 7.5) conditions, suggesting CIPK11 negatively regulates plasma membrane H^+-ATPase activity (Fuglsang *et al.*, 2007). Indeed, CIPK11 phosphorylates the AHA2 (ARABIDOPSIS H^+-ATPASE) at a defined site in the C-terminal autoinhibitory region and maintains it in a low-activity state (Fuglsang *et al.*, 2007), implicating the CBL-CIPK network in modulation of membrane potential. Concerning the CBL(s) involved in this process, CIPK11 was reported to specifically interact with CBL2 in protein-protein interaction assays, however there is no available genetic evidence linking CBL2 to CIPK11 in a functional context. Moreover, CBL2 specifically localizes to the tonoplast (Batistič *et al.*, 2012; Tang *et al.*, 2012) whereas AHA2 is located in the plasma membrane, leaving open the possibility that the observed interactions were nonspecific.

CBL-CIPK complexes may help direct the trafficking of integral membrane proteins. Recent evidence suggests CBL-CIPK pairs may regulate the subcellular distribution of MTPs. Arabidopsis CBL4/SOS3, which is a Type I CBL, and CIPK6, a putative interactor of CBL4, were shown to promote translocation of the potassium channel AKT2 from the ER to its site of activity at the plasma membrane. This function required dual lipid modifications to the N-terminus of CBL4 but, interestingly, did not require CIPK kinase activity (Held *et al.*, 2011). A previous study (Batistič *et al.*, 2008) showed by genetic ablation of the cysteine residue at position 3 of CBL1 that its plasma membrane-localization is dependent on S-acylation and that non-acylated CBL1 localizes to the ER. Hence, reversible S-acylation of this residue may direct the localization of Type I CBLs and their targets in a dynamic manner. Future work should investigate this possibility and focus on mechanistic links between CBL4 lipid modifications and AKT2 trafficking.

Some studies have indicated that CBLs may directly interact with target MTPs and possibly regulate their activities. One study (Grefen & Blatt, 2012) utilized a split-

ubiquitin Y2H system to show that the Type I CBLs 1,4, and 9 — but not the type II CBL6 —interacted directly with AKT1 in the absence of any CIPK. In plant cells, the Type I and Type II targeting motifs direct CBLs to the plasma membrane and vacuole, respectively. Considering that similar PTM machinery is present in yeast (Towler *et al.*, 1987; Roth *et al.*, 2002), it is quite possible that Type I CBLs efficiently target fusion proteins to the plasma membrane in yeast cells, whereas Type II CBLs do not and presumably localize elsewhere, such as the vacuole. Split-ubiquitin Y2H screens assay whether candidate proteins, which are fused to split-ubiquitin moieties, can facilitate their reconstitution and activate reporter gene expression. Similar to classical Y2H and bimolecular fluorescence complementation (BiFC) assays, the underlying principle of the split-ubiquitin assays lends itself vulnerable to false positives, and results must be viewed with caution. In particular, the results of Grefen & Blatt, (2012) could be alternatively attributed to colocalization of AKT1 and Type I CBLs to the plasma membrane, rather than bona fide protein-protein interactions; and further investigation will be needed to assess these possibilities.

Arabidopsis CBL10 was also reported to directly interact with AKT1 and negatively regulate its channel activity, possibly through competitive interactions with the CBL1/9-CIPK23 regulatory module (Ren *et al.*, 2013). Unlike previously identified AKT1 regulators, it appears that CBL10 is neither co-expressed in the same tissues nor targeted to the same subcellular localization (Kim *et al.*, 2007; Batistič *et al.*, 2010), although there has been some disagreement regarding the subcellular localization of CBL10. Importantly, the *cbl10* loss-of-function mutant did not show a discernible phenotype; therefore the phenotype of *CBL10* overexpression lines, which show hypersensitivity to low-potassium media, must be interpreted with caution. It can be difficult to discriminate physiologically relevant, direct effects from indirect effects, for example from misregulation of the expression or activity of other CBLs.

As previously mentioned, CaMs directly bind to a variety of MTPs and regulate their activities, therefore there is some precedent for direct interactions among calcium sensors and MTPs. Like CaMs, CBLs contain four EF-hands arranged in two pairs; however, unlike CaMs, the overall structure does not resemble a dumbbell and shares greater similarity with the calcineurin B subunit or neuronal calcium sensor (NCS) proteins. CBLs appear to specifically interact with CIPKs via the latter's conserved NAF motif and a CBL hydrophobic cleft (Shi *et al.*, 1999; Kim *et al.*, 2000; Albrecht *et al.*, 2001; Guo *et al.*, 2001). In contrast, the flexible linker region between the two pairs of EF-hands allows CaM to bind basic amphiphilic helices that can differ widely in sequence and properties (Crivici & Ikura, 1995). Whereas the N-terminal localization motifs of CBLs are critical for their functions, archetypal CaMs do not contain N-terminal localization motifs and sharply differ from CBLs in this respect. Based on canonical models, membrane-associated CBLs make direct physical contact with CIPKs that in turn target MTPs. Although CBLs may not directly bind MTPs, CBL lipid modifications may specifically guide CBL-CIPK pairs to the vicinity of their target MTPs, for example through association with lipid rafts; and this may be important for CBL-CIPK functions.

CBLs and CIPKs form a large and functionally interconnected signaling network positioned at various membranes within plant cells, and particular CBL-CIPK pairs have

been rigorously shown to modulate the activity of MTPs. The majority of published studies on CBL-CIPKs have focused on complexes with Type I CBLs that localize to the plasma membrane. Recent work has begun to elucidate the functions of tonoplast-localized Type II and Type III CBLs and partner CIPKs, however additional mechanistic insights and identification of target MTPs at the tonoplast are urgently needed. Moreover, possible CBL-CIPK functions elsewhere in the endomembrane system (e.g. the ER) should be investigated to provide a more complete perspective on plant membrane transport and signaling networks.

Looking Forward: Linking Transport Processes to Genes and Regulatory Networks.

The recent availability of genome sequence data for a breadth of different plants has enabled researchers to define the scope of plant transport systems at an unprecedented level and shed new light on classic plant electrophysiological studies from more than a decade earlier by revealing the genetic basis for observed currents and their associated ion fluxes. Phylogenomic inference has facilitated the identification and classification of MTPs and enabled predictions of their transport activities. Nonetheless, such predictions may be unreliable and must be corroborated by electrophysiological data, functional complementation, or alternative demonstration of transport activity.

Prior successes can be used to inform ongoing work on uncharacterized MTPs. Initial characterization of MTPs commonly intersects with gene expression analysis. In addition to tissue- or cell type-specific expression patterns, some MTPs are only expressed under specific conditions under which their transport activity is required. This phenomenon has been evidenced by studies on the potassium transporter HAK5 (Ahn *et al.*, 2004) and the magnesium transporter MGT6 (MAGNESIUM TRANSPORTER 6) (Mao *et al.*, 2014); each is required and transcriptionally induced upon low-substrate conditions. Downstream of transcriptional regulation, genes that encode MTPs, like other plant genes, typically contain multiple introns and must be spliced in order to produce mature transcripts. Predictive software has been widely implemented to predict exon-intron structure from genomic sequences, however these predictions can either be wrong or fail to account for multiple mature transcripts. Whereas PCR-cloning of transcripts from cDNA mitigates these concerns, DNA synthesis of predicted coding sequences (CDSs) has become increasingly popular as a facile and affordable alternative. However, particularly when used in conjunction with heterologous expression systems, reliance on predicted exon-intron structure can lead to spurious inferences. Although we do not know the extent to which alternative splicing leads to diverse protein isoforms from the same locus in plants, there is precedent for alternative splicing of MTPs from mammalian research (Tabuchi *et al.*, 2002; Denton *et al.*, 2004; Arniges *et al.*, 2006; Castiglioni *et al.*, 2006; Shen *et al.*, 2006).

Classic neurobiology works have further established that heteromeric complex formation among MTP subunits from distinct loci can be functionally important (Isacoff *et al.*, 1990; Sheng *et al.*, 1993; Krapivinsky *et al.*, 1995). The extent to which MTPs form

heteromeric complexes in plants is less understood, but several lines of evidence suggest it is common and widespread. Heteromeric MTP complexes may show profound discrepancies in transport activity compared to homomeric complexes made of constituent subunits. Silent subunits may modulate the transport activity of non-silent partner subunits, as has been shown in Arabidopsis for the silent, shaker-like potassium channel subunit KC1 in complex with other, non-silent shaker-like channel subunits, including AKT1 (Reintanz *et al.*, 2002; Duby *et al.*, 2008; Geiger *et al.*, 2009a; Jeanguenin *et al.*, 2011). Given that MTPs commonly function as multimeric complexes and can be comprised of heteromeric subunits, alternative splicing of MTP genes and assembly of MTPs from distinct loci could both be immensely important and warrant further investigation. Heterologous expression in *Xenopus* oocytes in conjunction with TEVC analysis has served as an important tool for investigating the functions of plant MTPs and can be adapted to effectively investigate multimeric MTPs. The procedure typically entails cloning a particular splice form of a transcript, transcribing it *in vitro*, and injecting the cRNA into oocytes, thereby prohibiting potential alternative splicing events that may contribute to assembly of a functional transport complex. In cases where multiple spliceforms have been predicted or detected, they could be tested combinatorially by co-injection of cRNAs into oocytes, as has been performed for heteromeric complexes encoded by distinct genes. Electrophysiological techniques for direct measurement of transport in plant cells remain challenging and are typically performed on intact protoplasts or detached segments of membrane. Nonetheless, such techniques, in conjunction with T-DNA insertional libraries in model plants like Arabidopsis, are powerful tools for loss-of-function analysis and provide more direct evidence of *in planta* activity than heterologous expression.

Subcellular localization of MTPs is functionally critical, and evaluation of MTP subcellular localization can facilitate downstream analyses. Whereas some MTP families appear to localize to particular cell membranes, other families show diverse localization patterns, and efforts to reliably predict the subcellular localization of MTPs through computational methods have heretofore been mostly unsuccessful. Proper localization of tagged MTP subunits may be predicated on their assembly into functional complexes with obligate partner subunits. Moreover, covalent attachment of fluorescent proteins or other tags can mask signal sequences and lead to aberrant localization patterns; and overexpression, which is commonly needed to visualize fluorescently tagged proteins, can also lead to artifacts in subcellular localization. For these reasons, functional complementation with tagged proteins provides the most compelling evidence for subcellular localization, however this is only feasible for a limited number of MTPs with defined phenotypes.

Despite its extensive utility in the study of membrane transport, approaches utilizing heterologous expression in oocytes or metazoan cells can be hampered by the absence of key regulators in foreign cells, therefore many inferred MTPs are electrically silent when heterologously expressed on their own. Identification of proteins that regulate MTPs can serve as a seminal step in the characterization of MTPs by facilitating electrophysiological analyses. This has been demonstrated for AKT1 in conjunction with its regulatory partners CBL1/9 and CIPK23 (Li *et al.*, 2006; Xu *et al.*,

2006) and the stomatal anion channel SLAC1 in conjunction with the protein kinase OST1 (OPEN STOMATA 1) (Geiger *et al.*, 2009b; Lee *et al.*, 2009) or other protein kinases (Maierhofer *et al.*, 2014). These successes highlight the value of functionally linking regulatory proteins and associated PTMs to known or predicted MTPs. Present and future challenges for plant membrane transport research include "forward" approaches that seek to identify the genetic basis for observed or inferred transport activities in an unbiased manner and "reverse" experimental approaches that can serve to validate and inform computational predictions.

In order to advance our understanding of the role of membrane transport in plant physiology and environmental responses, future studies should seek to place MTPs in the context of their native regulatory framework through a combination of approaches. Heterologous expression systems have proved to be useful tools in the identification and preliminary characterization of plant MTPs and their regulatory partners. Nonetheless, components may behave promiscuously in heterologous systems, and additional lines of evidence are required to disentangle physiologically relevant regulatory interactions from experimental artifacts. Loss-of-function genetic analysis serves as the most obvious and likely the most powerful means for validating inferred regulatory interactions and opening further avenues for research. For example, the CBL-CIPK-AKT1 regulatory cascade was characterized largely through molecular genetic analysis paired with electrophysiology, fluorescence microscopy, and biochemistry. Importantly, observations in heterologous systems (e.g. oocytes) were borne out by plant genetic analysis, as loss-of-function mutants in the pathway exhibited similar growth phenotypes to one another under low-potassium conditions. CRISPR-Cas technology has become a powerful tool for gene mutagenesis, especially for targeting multigene families, and will complement existing T-DNA insertional libraries in the functional analysis. For exceptionally large gene families that show functional redundancy, additional strategies are still needed to facilitate genetic analysis. Transcriptomic data is widely available and can be leveraged to identify genes encoding regulatory proteins and MTPs that show significant spatiotemporal overlap in their expression patterns and thereby narrow down targets for further investigation. Structural data is becoming increasingly available; and, notably, the crystal structures for a growing number of MTPs have been solved in recent years. Current research efforts should prioritize the identification of key regulatory sites on plant MTPs, such as residues subject to phosphoregulation, in order to complement structural analyses and to elucidate the mechanisms underlying known regulatory interactions. Coupled with advances in homology-based structural modeling, this vein of research will allow us to visualize the changes occurring at the molecular level that mediate the dynamic operations of membrane transport complexes. At the organismal level, 'omics-based technologies should continue to be harnessed for characterization of the transport and regulatory networks present in different parts of the plant, with the goal of mapping regulatory connections between these networks.

Conclusions.

Plant growth and metabolism are predicated on precise and continuous control over membrane transport. Membrane transport is regulated in diverse ways, providing the cell with the potential to regulate MTPs at each step from their synthesis to degradation. Plants exhibit elaborate transport systems that are specialized for different molecular species, yet transport of these distinct species is an interrelated process wherein small or seemingly unrelated perturbations can have pronounced, and often indirect, effects. Plants cope with these complexities and maintain effective membrane transport by integration of sensory inputs at signaling hubs; cytosolic calcium status serves as one such signal integration point and broadly directs various transport systems in a coordinated manner. By constantly adjusting their membrane transport systems, plants maintain homeostasis in shifting environments.

Sources Cited.

Ache P., Becker D., Ivashikina N., Dietrich P., Roelfsema M. R. G., & Hedrich R. (2000) GORK, a delayed outward rectifier expressed in guard cells of *Arabidopsis thaliana*, is a K^+-selective, K^+-sensing ion channel. *FEBS Letters* 486, 93-98.

Ahn S. J., Shin R., & Schachtman D. P. (2004) Expression of KT/KUP genes in Arabidopsis and the role of root hairs in K^+ uptake. *Plant Physiology* 134, 1135-45.

Akaboshi M., Hashimoto H., Ishida H., Saijo S., Koizumi N., Sato M., Shimizu T. (2008) The crystal structure of plant-specific calcium-binding protein AtCBL2 in complex with the regulatory domain of AtCIPK14. *Journal of Molecular Biology* 377, 246-57.

Albrecht V., Ritz O., Linder S., Harter K., & Kudla J. (2001) The NAF domain defines a novel protein–protein interaction module conserved in Ca^{2+}-regulated kinases. *The EMBO Journal* 20, 1051-63.

Allen G. J. & Sanders D. (1996) Control of ionic currents in guard cell vacuoles by cytosolic and luminal calcium. *The Plant Journal* 10, 1055-69.

Anisimova M. & Gascuel O. (2006) Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Systematic Biology* 55, 539-52.

Arniges M., Fernández-Fernández J. M., Albrecht N., Schaefer M., & Valverde M. A. (2006) Human TRPV4 channel splice variants revealed a key role of ankyrin domains in multimerization and trafficking. *Journal of Biological Chemistry* 281, 1580-86.

Barragán V., Leidi E. O., Andrés Z., Rubio L., De Luca A., Fernández J. A., Cubero B., & Pardo J. M. (2012) Ion exchangers NHX1 and NHX2 mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in Arabidopsis. *The Plant Cell* 24, 1127-42.

Batistič O., Sorek N., Schültke S., Yalovsky S., & Kudla J. (2008) Dual fatty acyl modification determines the localization and plasma membrane targeting of CBL/CIPK Ca^{2+} signaling complexes in Arabidopsis. *The Plant Cell* 20, 1346-62.

Batistič O. and Kudla J. (2009) Plant calcineurin B-like proteins and their interacting protein kinases. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1793, 985-92.

Batistič O., Waadt R., Steinhorst L., Held K., & Kudla J. (2010) CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. *The Plant Journal* 61, 211-22.

Batistič O. (2012) Genomics and localization of the Arabidopsis DHHC-cysteine-rich domain S-acyltransferase protein family. *Plant Physiology* 160, 1597-612.

Batistič O., Rehers M., Akerman A., Schlücking K., Steinhorst L., Yalovsky S., & Kudla J. (2012) S-acylation-dependent association of the calcium sensor CBL2 with the vacuolar membrane is essential for proper abscisic acid responses. *Cell Research* 22, 1155-68.

Bennett M.K., Calacos N., & Scheller R.H. (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* 257, 255-59.

Bethke P. C. & Jones R. L. (1994) Ca^{2+}-calmodulin modulates ion channel activity in storage protein vacuoles of barley aleurone cells. *The Plant Cell* 6, 277-85.

Bihler H., Eing C., Hebeisen S., Roller A., Czempinski K., & Bertl A. (2005) TPK1 is a vacuolar ion channel different from the slow-vacuolar cation channel. *Plant Physiology* 139, 417-24.

Blumwald E. & Poole R. J. (1985) Na^{+}/H^{+} antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. *Plant Physiology* 78, 163-67.

Bonza C., Carnelli A., De Michelis M. I., & Rasi-Caldogno F. (1998) Purification of the plasma membrane Ca^{2+}-ATPase from radish seedlings by calmodulin-agarose affinity chromatography. *Plant Physiology* 116, 845-51.

Brandt B., Brodsky D. E., Xue S., Negi J., Iba K., Kangasjarvi J., Ghassemian M., Stephan A. B., Hu H., & Schroeder J. I. (2012) Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. *Proceedings of the National Academy of Sciences, U.S.A.* 26, 10593-98.

Brett C. L., Donowitz M., & Rajini R. (2005) Evolutionary origins of eukaryotic sodium/proton exchangers. *American Journal of Physiology - Cell Physiology* 288, C223-39.

Caballero F., Botella M. A., Rubio L., Fernández J. A., Martínez V., & Rubio F. (2012) A Ca^{2+}-sensitive system mediates low-affinity K+ uptake in the absence of AKT1 in Arabidopsis plants. *Plant and Cell Physiology* 53, 2047-59.

Castiglioni A. J., Raingo J., & Lipscombe D. (2006) Alternative splicing in the C-terminus of CaV2.2 controls expression and gating of N-type calcium channels. *The Journal of Physiology* 576, 119-34.

Caterina M. J., Schumacher M. A., Tominaga M., Rosen T. A., Levine J. D., & Julius D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816-24.

Cellier F., Conéjéro G., Ricaud L., Luu D. T., Lepetit M., Gosti F., & Casse F. (2004) Characterization of AtCHX17, a member of the cation/H^+ exchangers, CHX family, from *Arabidopsis thaliana* suggests a role in K^+ homeostasis. *The Plant Journal* 39, 834-46.

Chanroj S., Lu Y., Padmanaban S., Nanatani K., Uozumi N., Rao R., & Sze H. (2011) Plant-specific cation/H^+ exchanger 17 and its homologs are endomembrane K^+ transporters with roles in protein sorting. *Journal of Biological Chemistry* 286, 33931-41.

Chatzigeorgiou M., Bang S., Hwang S. W., & Schafer W. R. (2013) tmc-1 encodes a sodium-sensitive channel required for salt chemosensation in *C. elegans*. *Nature* 494, 95-99.

Chaves-Sanjuan A., Sanchez-Barrena M. J., Gonzalez-Rubio J. M., Moreno M., Ragel P., Jimenez M., Pardo J. M., Martinez-Ripoll M., Quintero F. J., & Albert A. (2014) Structural basis of the regulatory mechanism of the plant CIPK family of protein kinases controlling ion homeostasis and abiotic stress. *Proceedings of the National Academy of Sciences, U.S.A.* 111, E4532-41.

Cheong Y. H., Pandey G. K., Grant J. J., Batistic O., Li L., Kim B., Lee S., Kudla J., & Luan S. (2007) Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. *The Plant Journal* 52, 223-39.

Choi W.-G., Toyota M., Kim S.-H., Hilleary R., & Gilroy S. (2014) Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. *Proceedings of the National Academy of Sciences, U.S.A.* 111, 6497-502.

Chung W. S., Lee S. H., Kim J. C., Do Heo W., Kim M. C., Park C. Y., Park H. C., Lim C. O., Kim W. B., & Harper J. F. (2000) Identification of a calmodulin-regulated soybean Ca^{2+}-ATPase (SCA1) that is located in the plasma membrane. *The Plant Cell* 12, 1393-407.

Clapham D. E. (2003) TRP channels as cellular sensors. *Nature* 426, 517-24.

Clapham D. E. (2007) Calcium signaling. *Cell* 131, 1047-58.

Crivici A. & Ikura M. (1995) Molecular and structural basis of target recognition by calmodulin. *Annual Review of Biophysics and Biomolecular Structure* 24, 85-116.

Czempinski K., Zimmermann S., Ehrhardt T., & Müller-Röber B. (1997) New structure and function in plant K^+ channels: KCO1, an outward rectifier with a steep Ca^{2+} dependency. *The EMBO Journal* 16, 2565-75.

Daram P., Urbach S., Gaymard F., Sentenac H., & Chérel I. (1997) Tetramerization of the AKT1 plant potassium channel involves its C-terminal cytoplasmic domain. *The EMBO journal* 16, 3455-63.

Davenport R. J., Muñoz-Mayor A., Jha D., Essah P. A., Rus A., & Tester M. (2007) The Na^+ transporter AtHKT1;1 controls retrieval of Na^+ from the xylem in Arabidopsis. *Plant, Cell & Environment* 30, 497-507.

De Silva D. L. R., Hetherington A. M., & Mansfield T. A. (1985) Synergism between calcium ions and abscisic acid in preventing stomatal opening. *New Phytologist* 100, 473-82.

Deeken R., Geiger D., Fromm J., Koroleva O., Ache P., Langenfeld-Heyser R., Sauer N., May S. T., & Hedrich R. (2002) Loss of the AKT2/3 potassium channel affects sugar loading into the phloem of Arabidopsis. *Planta* 216, 334-44.

Denton J., Nehrke K., Rutledge E., Morrison R., & Strange K. (2004) Alternative splicing of N-and C-termini of a *C. elegans* ClC channel alters gating and sensitivity to external Cl^- and H^+. *The Journal of Physiology* 555, 97-114.

Dreyer I., Antunes S., Hoshi T., Müller-Röber B., Palme K., Pongs O., Reintanz B., Hedrich R. (1997) Plant K^+ channel alpha-subunits assemble indiscriminately. *Biophysical Journal* 72, 2143-50.

Dreyer I., Michard E., Lacombe B., & Thibaud J.-B. (2001) A plant Shaker-like K^+ channel switches between two distinct gating modes resulting in either inward-rectifying or 'leak' current. *FEBS Letters* 505, 233-39.

Duby G., Hosy E., Fizames C., Alcon C., Costa A., Sentenac H., & Thibaud J. (2008) AtKC1, a conditionally targeted Shaker-type subunit, regulates the activity of plant K^+ channels. *The Plant Journal* 53, 115-23.

Dunkel M., Latz A., Schumacher K., Müller T., Becker D., & Hedrich R. (2008) Targeting of vacuolar membrane localized members of the TPK channel family. *Molecular Plant* 1, 938-49.

Durell S. R. & Guy H. R. (1999) Structural Models of the KtrB, TrkH, and Trk1, 2 Symporters Based on the Structure of the KcsA K^+ Channel. *Biophysical Journal* 77, 789-807.

Eisenach C., Chen Z. H., Grefen C., & Blatt M. R. (2012) The trafficking protein SYP121 of Arabidopsis connects programmed stomatal closure and K^+ channel activity with vegetative growth. *The Plant Journal* 69, 241-51.

Elumalai R. P., Nagpal P., & Reed J. W. (2002) A mutation in the Arabidopsis *KT2/KUP2* potassium transporter gene affects shoot cell expansion. *The Plant Cell* 14, 119-31.

Finka A., Cuendet A. F. H., Maathuis F. J. M., Saidi Y., & Goloubinoff P. (2012) Plasma membrane cyclic nucleotide gated calcium channels control land plant thermal sensing and acquired thermotolerance. *The Plant Cell* 24, 3333-48.

Fischer R. A. (1968) Stomatal opening: role of potassium uptake by guard cells. *Science* 160, 784-85.

Fuglsang A. T., Guo Y., Cuin T. A., Qiu Q., Song C., Kristiansen K. A., Bych K., Schulz A., Shabala S., & Schumaker K. S. (2007) Arabidopsis protein kinase PKS5 inhibits the plasma membrane H^+-ATPase by preventing interaction with 14-3-3 protein. *The Plant Cell* 19, 1617-34.

Furuichi T., Cunningham K. W., & Muto S. (2001) A putative two pore channel AtTPC1 mediates Ca^{2+} flux in Arabidopsis leaf cells. *Plant and Cell Physiology* 42, 900-5.

Gajdanowicz P., Michard E., Sandmann M., Rocha M., Corrêa L.G., Ramírez-Aguilar S.J., Gomez-Porras J.L., González W., Thibaud J.B., van Dongen J.T., Dreyer I. (2011) Potassium (K^+) gradients serve as a mobile energy source in plant vascular tissues. *Proceedings of the National Academy of Sciences, U.S.A.* 108, 864-69.

Garbarino J. & DuPont F. M. (1988) NaCl induces a Na^+/H^+ antiport in tonoplast vesicles from barley roots. *Plant Physiology* 86, 231-36.

Gaymard F., Pilot G., Lacombe B., Bouchez D., Bruneau D., Boucherez J., Michaux-Ferriere N., Thibaud J.-B., & Sentenac H. (1998) Identification and Disruption of a Plant Shaker-like Outward Channel Involved in K^+ Release into the Xylem Sap. *Cell* 94, 647-55.

Geiger D., Becker D., Vosloh D., Gambale F., Palme K., Rehers M., Anschuetz U., Dreyer I., Kudla J., & Hedrich R. (2009a) Heteromeric AtKC1· AKT1 channels in Arabidopsis roots facilitate growth under K^+-limiting conditions. *Journal of Biological Chemistry* 284, 21288-95.

Geiger D., Scherzer S., Mumm P., Stange A., Marten I., Bauer H., Ache P., Matschi S., Liese A., & Al-Rasheid K. A. S. (2009b) Activity of guard cell anion channel SLAC1 is

controlled by drought-stress signaling kinase-phosphatase pair. *Proceedings of the National Academy of Sciences, U.S.A.* 106, 21425-30.

Gerke V., Creutz C. E., & Moss S. E. (2005) Annexins: linking Ca^{2+} signalling to membrane dynamics. *Nature Reviews Molecular Cell Biology* 6, 449-61.

Gierth M., Mäser P., & Schroeder J.I. (2005) The potassium transporter *AtHAK5* functions in K^+ deprivation-induced high-affinity K^+ uptake and *AKT1* K^+ channel contribution to K^+ uptake kinetics in Arabidopsis roots. *Plant Physiology* 137, 1105-14.

Gobert A., Isayenkov S., Voelker C., Czempinski K., & Maathuis F. J. M. (2007) The two-pore channel *TPK1* gene encodes the vacuolar K^+ conductance and plays a role in K^+ homeostasis. *Proceedings of the National Academy of Sciences, U.S.A.* 104, 10726-31.

Gomez-Porras J. L., Riaño-Pachón D. M., Benito B., Haro R., Sklodowski K., Rodríguez-Navarro A., & Dreyer I. (2012) Phylogenetic analysis of K^+ transporters in bryophytes, lycophytes, and flowering plants indicates a specialization of vascular plants. *Frontiers in Plant Science* 3, 167.

Grefen C. & Blatt M. R. (2012) Do calcineurin B-like proteins interact independently of the serine threonine kinase CIPK23 with the K^+ channel AKT1? Lessons learned from a *menage a trois*. *Plant Physiology* 159, 915-19.

Guindon S., Delsuc F., Dufayard J. F., & Gascuel O. (2009) Estimating maximum likelihood phylogenies with PhyML. In *Bioinformatics for DNA Sequence Analysis* (ed. D. Posada), pp. 113-137. Springer, New York.

Guo Y., Halfter U., Ishitani M., & Zhu J.-K. (2001) Molecular characterization of functional domains in the protein kinase SOS2 that is required for plant salt tolerance. *The Plant Cell* 13, 1383-400.

Hall D., Evans A. R., Newbury H. J., & Pritchard J. (2006) Functional analysis of CHX21: a putative sodium transporter in Arabidopsis. *Journal of Experimental Botany* 57, 1201-10.

Harper J. F., Hong B., Hwang I., Guo H. Q., Stoddard R., Huang J. F., Palmgren M. G., & Sze H. (1998) A novel calmodulin-regulated Ca^{2+}-ATPase (ACA2) from Arabidopsis with an N-terminal autoinhibitory domain. *Journal of Biological Chemistry* 273, 1099-106.

Hassidim M., Braun Y., Lerner H. R., & Reinhold L. (1990) Na^+/H^+ and K^+/H^+ antiport in root membrane vesicles isolated from the halophyte *Atriplex* and the glycophyte cotton. *Plant Physiology* 94, 1795-801.

Held K., Pascaud F., Eckert C., Gajdanowicz P., Hashimoto K., Corratgé-Faillie C., Offenborn J. N., Lacombe B., Dreyer I., & Thibaud J.-B. (2011) Calcium-dependent modulation and plasma membrane targeting of the AKT2 potassium channel by the CBL4/CIPK6 calcium sensor/protein kinase complex. *Cell Research* 21, 1116-30.

Hemsley P. A. & Grierson C. S. (2008) Multiple roles for protein palmitoylation in plants. *Trends in Plant Science* 13, 295-302.

Hirsch R. E., Lewis B. D., Spalding E. P., & Sussman M. R. (1998) A role for the AKT1 potassium channel in plant nutrition. *Science* 280, 918-21.

Ho C. H., Lin S. H., Hu H. C., & Tsay Y. F. (2009) CHL1 functions as a nitrate sensor in plants. *Cell* 138, 1184-94.

Honsbein A., Sokolovski S., Grefen C., Campanoni P., Pratelli R., Paneque M., Chen Z., Johansson I., & Blatt M. R. (2009) A tripartite SNARE-K+ channel complex mediates in channel-dependent K^+ nutrition in Arabidopsis. *The Plant Cell* 21, 2859-77.

Horie T., Brodsky D. E., Costa A., Kaneko T., Schiavo F. L., Katsuhara M., & Schroeder J. I. (2011) K^+ transport by the OsHKT2; 4 transporter from rice with atypical Na^+ transport properties and competition in permeation of K^+ over Mg^{2+} and Ca^{2+} ions. *Plant Physiology* 156, 1493-507.

Hosy E., Vavasseur A., Mouline K., Dreyer I., Gaymard F., Porée F., Boucherez J., Lebaudy A., Bouchez D., & Véry A.-A. (2003) The Arabidopsis outward K^+ channel GORK is involved in regulation of stomatal movements and plant transpiration. *Proceedings of the National Academy of Sciences, U.S.A.* 100, 5549-54.

Hou C., Tian W., Kleist T., He K., Garcia V., Bai F., Hao Y., Luan S., & Li L. (2014) DUF221 proteins are a family of osmosensitive calcium-permeable cation channels conserved across eukaryotes. *Cell Research* 24, 632-35.

Hrabak E. M., Chan C. W., Gribskov M., Harper J. F., Choi J. H., Halford N., Kudla J., Luan S., Nimmo H. G., Sussman M. R., Thomas M., Walker-Simmons K., Zhu J. K., & Harmon A. C. (2003) The Arabidopsis CDPK-SnRK superfamily of protein kinases. *Plant Physiology* 132, 666-80.

Humble G. D. & Hsiao T. C. (1970) Light-dependent influx and efflux of potassium of guard cells during stomatal opening and closing. *Plant Physiology* 46, 483-87.

Hwang I., Sze H., & Harper J. F. (2000) A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca^{2+} pump (ACA2) located in the endoplasmic reticulum of Arabidopsis. *Proceedings of the National Academy of Sciences, U.S.A.* 97, 6224-29.

Isacoff E. Y., Jan Y. N., & Jan L. Y. (1990) Evidence for the formation of heteromultimeric potassium channels in *Xenopus* oocytes. *Nature* 345, 530-34.

Jeanguenin L., Alcon C., Duby G., Boeglin M., Chérel I., Gaillard I., Zimmermann S., Sentenac H., & Véry A. (2011) AtKC1 is a general modulator of Arabidopsis inward Shaker channel activity. *The Plant Journal* 67, 570-82.

Katoh K., Kuma K., & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res*earch 30, 3059–66.

Kelley L. A. & Sternberg M. J. E. (2009) Protein structure prediction on the Web: a case study using the Phyre server. *Nature Protocols* 4, 363-71.

Kim B. G., Waadt R., Cheong Y. H., Pandey G. K., Dominguez-Solis J. R., Schultke S., Lee S. C., Kudla J., & Luan S. (2007) The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabidopsis. *The Plant Journal* 52, 473-84.

Kim K.-N., Cheong Y. H., Gupta R., & Luan S. (2000) Interaction specificity of Arabidopsis calcineurin B-like calcium sensors and their target kinases. *Plant Physiology* 124, 1844-53.

Kleist T. J., Spencley A. L., & Luan S. (2014) Comparative phylogenomics of the CBL-CIPK calcium-decoding network in the moss *Physcomitrella*, Arabidopsis, and other green lineages. *Frontiers in Plant Science* 5, 187.

Knight H., Trewavas A. J., & Knight M. R. (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *The Plant Journal* 12, 1067-78.

Knight M. R., Campbell A. K., Smith S. M., & Trewavas A. J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352, 524-526.

Kohler C., Merkle T., & Neuhaus G. (1999) Characterisation of a novel gene family of putative cyclic nucleotide- and calmodulin-regulated ion channels in *Arabidopsis thaliana*. *The Plant Journal* 18, 97-104.

Krapivinsky G., Gordon E. A., Wickman K., Velimirovic B., Krapivinsky L., & Clapham D. E. (1995) The G-protein-gated atrial K^+ channel IKACh is a heteromultimer of two inwardly rectifying K^+-channel proteins. *Nature* 374, 135-41.

Kurima K., Peters L. M., Yang Y., Riazuddin S., Ahmed Z. M., Naz S., Arnaud D., Drury S., Mo J., & Makishima T. (2002) Dominant and recessive deafness caused by

mutations of a novel gene, *TMC1*, required for cochlear hair-cell function. *Nature Genetics* 30, 277-84.

Lacombe B., Pilot G., Michard E., Gaymard F., Sentenac H., & Thibaud J.-B. (2000) A shaker-like K^+ channel with weak rectification is expressed in both source and sink phloem tissues of Arabidopsis. *The Plant Cell* 12, 837-51.

Lam H.-M., Chiu J., Hsieh M.-H., Meisel L., Oliveira I. C., Shin M., & Coruzzi G. (1998) Glutamate-receptor genes in plants. *Nature* 396, 125-26.

Lan W.-Z., Lee S.-C., Che Y.-F., Jiang Y.-Q., & Luan S. (2011) Mechanistic analysis of AKT1 regulation by the CBL–CIPK–PP2CA interactions. *Molecular Plant* 4, 527-36.

Lan W. Z., Wang W., Wang S. M., Li L. G., Buchanan B. B., Lin H. X., Gao J. P., & Luan S. (2010) A rice high-affinity potassium transporter (HKT) conceals a calcium-permeable cation channel. *Proceedings of the National Academy of Sciences, U.S.A.* 107, 7089-94.

Laohavisit A., Shang Z., Rubio L., Cuin T. A., Véry A.-A., Wang A., Mortimer J. C., Macpherson N., Coxon K. M., & Battey N. H. (2012) Arabidopsis annexin1 mediates the radical-activated plasma membrane Ca^{2+}-and K^+-permeable conductance in root cells. *The Plant Cell* 24, 1522-33.

Lebaudy A., Vavasseur A., Hosy E., Dreyer I., Leonhardt N., Thibaud J.-B., Véry A.-A., Simonneau T., & Sentenac H. (2008) Plant adaptation to fluctuating environment and biomass production are strongly dependent on guard cell potassium channels. *Proceedings of the National Academy of Sciences, U.S.A.* 105, 5271-76.

Lee S. C., Lan W.-Z., Kim B.-G., Li L., Cheong Y. H., Pandey G. K., Lu G., Buchanan B. B., & Luan S. (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. *Proceedings of the National Academy of Sciences, U.S.A.* 104, 15959-64.

Lee S. C., Lan W., Buchanan B. B., & Luan S. (2009) A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. *Proceedings of the National Academy of Sciences, U.S.A.* 106, 21419-24.

Lehti-Shiu M. D. & Shiu S.-H. (2012) Diversity, classification and function of the plant protein kinase superfamily. *Philosophical Transactions of the Royal Society B: Biological Sciences* 367, 2619-39.

Leng Q., Mercier R. W., Yao W., & Berkowitz G. A. (1999) Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. *Plant Physiology* 121, 753-61.

Li J., Lee Y. R., & Assmann S. M. (1998) Guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 potassium channel. *Plant Physiology* 116, 785-95.

Li L., Kim B.-G., Cheong Y. H., Pandey G. K., & Luan S. (2006) A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in Arabidopsis. *Proceedings of the National Academy of Sciences, U.S.A.* 103, 12625-30.

Li L., Liu K., Hu Y., Li D., & Luan S. (2008) Single mutations convert an outward K^+ channel into an inward K^+ channel. *Proceedings of the National Academy of Sciences, U.S.A.* 105, 2871-76.

Liu H., Tang R., Zhang Y. U. E., Wang C., Lv Q., Gao X., Li W., & Zhang H. (2010) AtNHX3 is a vacuolar K^+/H^+ antiporter required for low-potassium tolerance in *Arabidopsis thaliana*. *Plant, Cell & Environment* 33, 1989-99.

Liu K., Li L., & Luan S. (2006) Intracellular K^+ sensing of SKOR, a Shaker-type K+ channel from Arabidopsis. *The Plant Journal* 46, 260-68.

Luan S. (2009) The CBL-CIPK network in plant calcium signaling. *Trends in Plant Science* 14, 37-42.

Maathuis F.J.M. & Amtmann A. (1999) K^+ Nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. *Annals of Botany* 84, 123-33.

Maierhofer T., Diekmann M., Offenborn J.N., Lind C., Bauer H., Hashimoto K., S Al-Rasheid K.A., Luan S., Kudla J., Geiger D., Hedrich R. (2014) Site- and kinase-specific phosphorylation-mediated activation of SLAC1, a guard cell anion channel stimulated by abscisic acid. *Science Signaling* 7, ra86.

Mao D., Chen J., Tian L., Liu Z., Yang L., Tang R., Li J., Lu C., Yang Y., Shi J., Chen L., Li D., & Luan S. (2014) Arabidopsis Transporter MGT6 Mediates Magnesium Uptake and Is Required for Growth under Magnesium Limitation. *The Plant Cell* 26, 2234-48.

Martins T. V., Evans M. J., Woolfenden H. C., & Morris R. J. (2013) Towards the physics of calcium signalling in plants. *Plants* 2, 541-88.

McAinsh M. R. (1990) Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. *Nature* 343, 186-88.

Michard E., Dreyer I., Lacombe B., Sentenac H., & Thibaud J. (2005) Inward rectification of the AKT2 channel abolished by voltage-dependent phosphorylation. *The Plant Journal* 44, 783-97.

Moorhead G. B., De Wever V., Templeton G., & Kerk D. (2009) Evolution of protein phosphatases in plants and animals. *Biochemical Journal* 417, 401-9.

Mori I. C., Murata Y., Yang Y., Munemasa S., Wang Y. F., Andreoli S., Tiriac H., Alonso J. M., Harper J. F., Ecker J. R., Kwak J. M., & Schroeder J. I. (2006) CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. *PLoS Biology* 4, e327.

Mousavi S. A. R., Chauvin A., Pascaud F., Kellenberger S., & Farmer E. E. (2013) GLUTAMATE RECEPTOR-LIKE genes mediate leaf-to-leaf wound signalling. *Nature* 500, 422-26.

Nagae M., Nozawa A., Koizumi N., Sano H., Hashimoto H., Sato M., & Shimizu T. (2003) The crystal structure of the novel calcium-binding protein AtCBL2 from *Arabidopsis thaliana*. *Journal of Biological Chemistry* 278, 42240-46.

Nakagawa Y., Katagiri T., Shinozaki K., Qi Z., Tatsumi H., Furuichi T., Kishigami A., Sokabe M., Kojima I., & Sato S. (2007) Arabidopsis plasma membrane protein crucial for Ca^{2+} influx and touch sensing in roots. *Proceedings of the National Academy of Sciences, U.S.A.* 104, 3639-44.

O'Rourke S. M. & Herskowitz I. (2002) A third osmosensing branch in *Saccharomyces cerevisiae* requires the Msb2 protein and functions in parallel with the Sho1 branch. *Molecular and Cellular Biology* 22, 4739-49.

Ohta M., Guo Y., Halfter U., & Zhu J.-K. (2003) A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. *Proceedings of the National Academy of Sciences, U.S.A.* 100, 11771-76.

Osakabe Y., Yamaguchi-Shinozaki K., Shinozaki K., & Tran L. S. (2013) Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress. *Journal of Experimental Botany* 64, 445-58.

Pan B., Géléoc G. S., Asai Y., Horwitz G. C., Kurima K., Ishikawa K., Kawashima Y., Griffith A. J., & Holt J. R. (2013) TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. *Neuron* 79, 504-15.

Peier A. M., Moqrich A., Hergarden A. C., Reeve A. J., Andersson D. A., Story G. M., Earley T. J., Dragoni I., McIntyre P., Bevan S., & Patapoutian A. (2002) A TRP channel that senses cold stimuli and menthol. *Cell* 108, 705-15.

Peiter E., Maathuis F. J. M., Mills L. N., Knight H., Pelloux J., Hetherington A. M., & Sanders D. (2005) The vacuolar Ca^{2+}-activated channel TPC1 regulates germination and stomatal movement. *Nature* 434, 404-8.

Pettersen E. F., Goddard T. D., Huang C. C., Couch G. S., Greenblatt D. M., Meng E. C., & Ferrin T. E. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *Journal of Computational Chemistry* 25, 1605-12.

Pilot G., Lacombe B., Gaymard F., Chérel I., Boucherez J., Thibaud J.-B., & Sentenac H. (2001) Guard Cell Inward K^+ Channel Activity in Arabidopsis Involves Expression of the Twin Channel Subunits KAT1 and KAT2. *Journal of Biological Chemistry* 276, 3215-21.

Porée F., Wulfetange K., Naso A., Carpaneto A., Roller A., Natura G., Bertl A., Sentenac H., Thibaud J.B., & Dreyer I. (2005) Plant K(in) and K(out) channels: approaching the trait of opposite rectification by analyzing more than 250 KAT1-SKOR chimeras. *Biochemical and Biophysical Research Communications* 332, 465-73.

Pyo Y.J., Gierth M., Schroeder J.I., & Cho M.H. (2010) High-affinity K^+ transport in Arabidopsis: AtHAK5 and AKT1 are vital for seedling establishment and postgermination growth under low-potassium conditions. *Plant Physiology* 153, 863-75.

Qiu Q.-S., Guo Y., Dietrich M. A., Schumaker K. S., & Zhu J.-K. (2002) Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. *Proceedings of the National Academy of Sciences, U.S.A.* 99, 8436-41.

Quan R., Lin H., Mendoza I., Zhang Y., Cao W., Yang Y., Shang M., Chen S., Pardo J. M., & Guo Y. (2007) SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress. *The Plant Cell* 19, 1415-31.

Quintero F. J., Ohta M., Shi H., Zhu J.-K., & Pardo J. M. (2002) Reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na^+ homeostasis. *Proceedings of the National Academy of Sciences, U.S.A.* 99, 9061-66.

Quintero F. J., Martinez-Atienza J., Villalta I., Jiang X., Kim W.-Y., Ali Z., Fujii H., Mendoza I., Yun D.-J., & Zhu J.-K. (2011) Activation of the plasma membrane Na/H antiporter Salt-Overly-Sensitive 1 (SOS1) by phosphorylation of an auto-inhibitory C-terminal domain. *Proceedings of the National Academy of Sciences, U.S.A.* 108, 2611-16.

Reintanz B., Szyroki A., Ivashikina N., Ache P., Godde M., Becker D., Palme K., & Hedrich R. (2002) AtKC1, a silent Arabidopsis potassium channel α-subunit modulates

root hair K^+ influx. *Proceedings of the National Academy of Sciences, U.S.A.* 99, 4079-84.

Ren X., Qi G., Feng H., Zhao S., Zhao S., Wang Y., & Wu W. (2013) Calcineurin B-like protein CBL10 directly interacts with AKT1 and modulates K^+ homeostasis in Arabidopsis. *The Plant Journal* 74, 258-66.

Richards S. L., Laohavisit A., Mortimer J. C., Shabala L., Swarbreck S. M., Shabala S., & Davies J. M. (2014) Annexin 1 regulates the H_2O_2-induced calcium signature in *Arabidopsis thaliana* roots. *The Plant Journal* 77, 136-45.

Riedelsberger J., Sharma T., Gonzalez W., Gajdanowicz P., Morales-Navarro S.E., Garcia-Mata C., Mueller-Roeber B., González-Nilo F.D. Blatt M.R., & Dreyer I. (2010) Distributed structures underlie gating differences between the K_{in} Channel KAT1 and the K_{out} channel SKOR. *Molecular Plant* 3, 236-45.

Rigas S., Debrosses G., Haralampidis K., Vicente-Agullo F., Feldmann K. A., Grabov A., Dolan L., & Hatzopoulos P. (2001) *TRH1* encodes a potassium transporter required for tip growth in Arabidopsis root hairs. *The Plant Cell* 13, 139-51.

Rodriguez L., Gonzalez-Guzman M., Diaz M., Rodrigues A., Izquierdo-Garcia A. C., Peirats-Llobet, Fernandez M. A., Antoni R., Fernandez D., Marquez J. A., Mulet J. M., Albert A., & Rodriguez P. L. (2014). C2-domain abscisic acid-related proteins mediate the interaction of PYR/PYL/RCAR abscisic acid receptors with the plasma membrane and regulate abscisic acid sensitivity in Arabidopsis. *The Plant Cell*, *26*, 4802-4820.

Roth A. F., Feng Y., Chen L., & Davis N. G. (2002) The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. *Journal of Cell Biology* 159, 23-28.

Rubio F., Gassmann W., & Schroeder J. I. (1995) Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. *Science* 270, 1660-63.

Rubio F., Nieves-Cordones M., Alemán F., & Martínez V. (2008) Relative contribution of AtHAK5 and AtAKT1 to K^+ uptake in the high-affinity range of concentrations. *Physiologia Plantarum* 134, 598-608.

Rubio F., Alemán F., Nieves-Cordones M., & Martínez V. (2010) Studies on Arabidopsis *athak5*, *atakt1* double mutants disclose the range of concentrations at which AtHAK5, AtAKT1 and unknown systems mediate K^+ uptake. *Physiologia Plantarum* 139, 220-28.

Rubio F., Fon M., Ródenas R., Nieves-Cordones M., Alemán F., Rivero R. M., & Martínez V. (2014) A low K^+ signal is required for functional high-affinity K^+ uptake through HAK5 transporters. *Physiologia Plantarum* 152, 558-70.

Saimi Y. & Kung C. (2002) Calmodulin as an ion channel subunit. *Annual Review of Physiology* 64, 289-311.

Sánchez-Barrena M.J., Martínez-Ripoli M., Zhu J.-K., & Albert A. (2005) The structure of the *Arabidopsis thaliana* SOS3: molecular mechanism of sensing calcium for salt stress response. *Journal of Molecular Biology* 345, 1253-64.

Sánchez-Barrena M.J., Fujii H., Angulo I., Martínez-Ripoli M., Zhu J.-K., & Albert A. (2007) The structure of the protein kinase AtSOS2 bound to the calcium sensor AtSOS3. *Molecular Cell* 26, 427-435.

Schachtman D. P. & Schroeder J. I. (1994) Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. *Nature* 370, 655-658.

Scherzer S., Maierhofer T., Al-Rasheid K. A. S., Geiger D., & Hedrich R. (2012) Multiple calcium-dependent kinases modulate ABA-activated guard cell anion channels. *Molecular Plant* 5, 1409-12.

Schroeder J. I. & Hagiwara S. (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature* 338, 427-30.

Schuurink R. C., Shartzer S. F., Fath A., & Jones R. L. (1998) Characterization of a calmodulin-binding transporter from the plasma membrane of barley aleurone. *Proceedings of the National Academy of Sciences, U.S.A.* 95, 1944-49.

Shabala S., Demidchik V., Shabala L., Cuin T. A., Smith S. J., Miller A. J., Davies J. M., & Newman I. A. (2006) Extracellular Ca^{2+} ameliorates NaCl-induced K^+ loss from Arabidopsis root and leaf cells by controlling plasma membrane K^+-permeable channels. *Plant Physiology* 141, 1653-65.

Shabala S. & Cuin T. A. (2008) Potassium transport and plant salt tolerance. *Physiologia Plantarum* 133, 651-69.

Shao X., Li C., Fernandez I., Zhang X., Südhof T.C., & Rizo J. (1997) Synaptotagmin-syntaxin interaction: the C2 domain as a Ca^{2+}-dependent electrostatic switch. *Neuron* 18, 133-42.

Shen Y., Yu D., Hiel H., Liao P., Yue D. T., Fuchs P. A., & Soong T. W. (2006) Alternative splicing of the Cav1.3 channel IQ domain, a molecular switch for Ca^{2+}-dependent inactivation within auditory hair cells. *The Journal of Neuroscience* 26, 10690-99.

Sheng M., Liao Y. J., Jan Y. N., & Jan L. Y. (1993) Presynaptic A-current based on heteromultimeric K^+ channels detected *in vivo*. *Nature* 365, 72-75.

Shi J., Kim K.-N., Ritz O., Albrecht V., Gupta R., Harter K., Luan S., & Kudla J. (1999) Novel protein kinases associated with calcineurin B–like calcium sensors in Arabidopsis. *The Plant Cell* 11, 2393-405.

Siegel N. & Hang A. (1983) Calmodulin dependent formation of membrane potential in barley root plasma membrane vesicles: A biochemical model of aluminum toxicity in plants. *Physiologia Plantarum* 59, 285-91.

Singh A., Giri J., Kapoor S., Tyagi A. K., & Pandey G. K. (2010) Protein phosphatase complement in rice: genome-wide identification and transcriptional analysis under abiotic stress conditions and reproductive development. *BMC Genomics* 11, 435.

Spalding E.P., Hirsch R.E., Lewis D.R., Qi Z., Sussman M.R., & Lewis B.D. (1999) Potassium uptake supporting plant growth in the absence of AKT1 channel activity: inhibition by ammonium and stimulation by sodium. *Journal of General Physiology* 113, 909-18.

Sutter J.-U., Sieben C., Hartel A., Eisenach C., Thiel G., & Blatt M. R. (2007) Abscisic Acid Triggers the Endocytosis of the *Arabidopsis* KAT1 K^+ Channel and Its Recycling to the Plasma Membrane. *Current Biology* 17, 1396-402.

Sze H., Geisler M., & Murphy A. S. (2013) Linking the evolution of plant transporters to their functions. *Frontiers in Plant Science* 4, 547.

Szyroki A., Ivashikina N., Dietrich P., Roelfsema M. R. G., Ache P., Reintanz B., Deeken R., Godde M., Felle H., & Steinmeyer R. (2001) KAT1 is not essential for stomatal opening. *Proceedings of the National Academy of Sciences* 98, 2917-21.

Tabuchi M., Tanaka N., Nishida-Kitayama J., Ohno H., & Kishi F. (2002) Alternative splicing regulates the subcellular localization of divalent metal transporter 1 isoforms. *Molecular Biology of the Cell* 13, 4371-87.

Tang R.-J., Liu H., Yang Y., Yang L., Gao X.-S., Garcia V. J., Luan S., & Zhang H.-X. (2012) Tonoplast calcium sensors CBL2 and CBL3 control plant growth and ion homeostasis through regulating V-ATPase activity in Arabidopsis. *Cell Research* 22, 1650-65.

Tang R.-J., Yang Y., Yang L., Liu H., Wang C.-T., Yu M.-M., Gao X.-S., & Zhang H.-X. (2014) Poplar calcineurin B-like proteins PtCBL10A and PtCBL10B regulate shoot salt tolerance through interaction with PtSOS2 in the vacuolar membrane. *Plant, Cell & Environment* 37, 573-88.

Tang R.-J., Zhao F.-G., Garcia V.J., Kleist T.J., Yang L., Zhang H.X., & Luan S. (2015) Tonoplast CBL-CIPK calcium signaling network regulates magnesium homeostasis in Arabidopsis. *Proceedings of the National Academy of Sciences U.S.A* 112, 3134-39.

Tatebayashi K., Tanaka K., Yang H., Yamamoto K., Matsushita Y., Tomida T., Imai M., & Saito H. (2007) Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *The EMBO Journal* 26, 3521-33.

Towler D. A., Eubanks S. R., Towery D. S., Adams S. P., & Glaser L. (1987) Amino-terminal processing of proteins by N-myristoylation. Substrate specificity of N-myristoyl transferase. *Journal of Biological Chemistry* 262, 1030-36.

Venema K., Quintero F. J., Pardo J. M., & Donaire J. P. (2002) The Arabidopsis Na^+/H^+ exchanger AtNHX1 catalyzes low affinity Na^+ and K^+ transport in reconstituted liposomes. *Journal of Biological Chemistry* 277, 2413-18.

Venema K., Belver A., Marín-Manzano M. C., Rodríguez-Rosales M. P., & Donaire J. P. (2003) A novel intracellular K^+/H^+ antiporter related to Na^+/H^+ antiporters is important for K^+ ion homeostasis in plants. *Journal of Biological Chemistry* 278, 22453-59.

Vicente-Agullo F., Rigas S., Desbrosses G., Dolan L., Hatzopoulos P., & Grabov A. (2004) Potassium carrier TRH1 is required for auxin transport in Arabidopsis roots. *The Plant Journal* 40, 523-35.

Wang Y. & Wu W.-H. (2013) Potassium transport and signaling in higher plants. *Annual Review of Plant Biology* 64, 451-76.

Ward J. M. & Schroeder J. I. (1994) Calcium-activated K^+ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *The Plant Cell* 6, 669-83.

Xu J., Li H.-D., Chen L.-Q., Wang Y., Liu L.-L., He L., & Wu W.-H. (2006) A Protein Kinase, Interacting with Two Calcineurin B-like Proteins, Regulates K^+ Transporter AKT1 in *Arabidopsis*. *Cell* 125, 1347-60.

Yamanaka T., Nakagawa Y., Mori K., Nakano M., Imamura T., Kataoka H., Terashima A., Iida K., Kojima I., & Katagiri T. (2010) MCA1 and MCA2 that mediate Ca^{2+} uptake have distinct and overlapping roles in Arabidopsis. *Plant Physiology* 152, 1284-96.

Yuan F., Yang H., Xue Y., Kong D., Ye R., Li C., Zhang J., Theprungsirikul L., Shrift T., Krichilsky B., Johnson D. M., Swift G. B., He Y., Siedow J. N., & Pei Z.-M. (2014) OSCA1 mediates osmotic-stress-evoked Ca^{2+} increases vital for osmosensing in Arabidopsis. *Nature* 514, 367-71.

Zhao J., Cheng N.-H., Motes C. M., Blancaflor E. B., Moore M., Gonzales N., Padmanaban S., Sze H., Ward J. M., & Hirschi K. D. (2008) AtCHX13 is a plasma membrane K^+ transporter. *Plant Physiology* 148, 796-807.

Zozulya S. & Stryer L. (1992) Calcium-myristoyl protein switch. *Proceedings of the National Academy of Sciences, U.S.A.* 89, 11569-73.

Chapter 3: Phylogenomic classification of calcineurin B-like calcium sensors and associated protein kinases in land plants and green algae.

The following chapter (excluding the preface) was published as a peer-reviewed research article in the journal *Frontiers in Plant Science* and is reproduced here with minor editorial changes. Copyright is retained by the authors.

Kleist T.J., Spencley A.L., & Luan, S. (2014) Comparative phylogenomics of the CBL-CIPK calcium-decoding network in the moss *Physcomitrella*, Arabidopsis, and other green lineages. *Frontiers in Plant Science* 5, 187.

Contributions.

T.J. Kleist planned and conducted experiments, interpreted results, prepared figures, and wrote the manuscript. A.L. Spencley served as an undergraduate research assistant under the mentorship of T.J. Kleist, and he helped to conduct experiments, interpret results, and finalize the manuscript. S. Luan helped to plan experiments, interpret results, and edit the manuscript.

Preface.

The molecular machinery for encoding and decoding calcium signals in plants appears to be quite ancient and likely played a critical role in the colonization of land. The previous chapter examined the role of calcium signaling in regulating membrane transport processes. Briefly, CBL-type calcium sensors and CIPKs – their associated protein kinases – form an interconnected signaling network that interfaces among various membrane transport proteins to coordinate transport processes in plants. The CBL-CIPK network has been exclusively investigated in Arabidopsis and other flowering plants, however bioinformatic analyses have suggested that the CBL-CIPK signaling network is present among early-diverging land plant lineages and certain green algae. The following chapter provides a computational classification of CBLs and CIPKs guided by phylogenomics and summarizes efforts to characterize CBL and CIPK homologs in the moss *Physcomitrella patens*. Computationally predicted interactions among *Physcomitrella* CBL-CIPK pairs were validated using yeast two-hybrid analyses, supporting the hypothesis that CBLs and CIPKs form an interconnected signaling network in early-diverging land plants much like their homologs from flowering plants. Data described here suggest that CBL N-terminal localization signals, which target CBL-CIPK pairs to cellular membranes, diversified during the course of land plant evolution. Among algal CBLs, only a single type of localization motif was identified, and this motif triggers plasma membrane targeting in flowering plants. Hence, diversification of N-terminal localization motifs may have allowed CBL-CIPK pairs to target membrane transport proteins at both endomembranes and the plasma membrane.

Introduction.

Of the events that have shaped our modern biosphere, the colonization of land by the predecessors of modern embryophytes stands out as an evolutionary advent that has profoundly affected our landscape and terrestrial ecology. Land plants arose roughly 450 million years ago from a lineage of multicellular freshwater green algae known as charophytes (Graham 1996; Lewis & McCourt 2004). Land plants have elaborated and expanded upon a molecular toolkit present in their charophyte ancestors and thereby developed novel anatomical and molecular adaptations to sustain life on land (Graham 1996; Kenrick & Crane 1997; Pittermann 2010; Timme & Delwiche 2010). The switch from aquatic to terrestrial growth imposed new and formidable abiotic stresses. Discontinuous access to water combined with labile, often unfavorable ion balances spurred the development of sophisticated mechanisms for responses to water and ion availability, the communication of this information throughout the plant body, and the coordination of orchestrated responses to these stresses.

Calcium ions play a pivotal role in a host of signal transduction cascades in plants as well as in animals. Tightly localized spikes in cytosolic calcium concentration in response to particular environmental cues have been extensively documented in plant cells and are thought to act as early steps in plant signaling pathways (Gilroy *et al.* 1993; Evans *et al.* 2001). These bursts, known as calcium signals, are modulated by channels that allow calcium entry from both outside the cell and inside cellular stores (e.g. the vacuole, endoplasmic reticulum). Calcium signals are decoded by proteins that act as sensors; calcium sensors often contain helix-loop-helix motifs known as EF hands that bind calcium and induce conformational changes to modulate the activity of other proteins or domains (Hrabak *et al.* 2003; McCormack *et al.* 2005).

Calcineurin B-Like proteins or CBLs are a family of calcium sensors found in all studied land plants and some chlorophyte green algae (Weinl & Kudla 2009; Batistic *et al.* 2011). CBLs are named based on their homology to the B regulatory subunit of the phosphatase calcineurin (Luan *et al.* 2002). CBLs contain four calcium-binding EF hands and typically contain a subcellular localization signal at their N-terminus. The most thoroughly characterized CBLs to date contain a dual lipid modification motif (MGCXXS/T) at their N-terminus that is necessary and sufficient for targeting of fluorescent protein (FP)-fusions to the plasma membrane (Batistič *et al.* 2008, 2010). Other CBLs are reported to localize to the vacuole, and several of these CBLs contain a distinct N-terminal extension known as the Tonoplast Targeting Sequence (TTS) that targets FP-fusions to the tonoplast (Batistič *et al.* 2012; Tang *et al.* 2012). Uniquely, Arabidopsis CBL10 contains a putative N-terminal transmembrane helix that anchors it to the tonoplast (Kim *et al.* 2007; Batistič *et al.* 2010) or plasma membrane (Quan *et al.* 2007; Ren *et al.* 2013). Subcellular targeting has been shown to be critical for CBL functionality, and CBLs are responsible for the recruitment and localization of protein partners.

CBLs physically and functionally interact with CBL-Interacting Protein Kinases (CIPKs) and modulate their kinase activity (Shi *et al.* 1999; Batistic *et al.* 2011). Hence, the CBL-CIPK network serves to decode calcium signals and transmit these signals

through reversible protein phosphorylation. CIPKs, also known as SnRK3 proteins, are serine/threonine protein kinases that consist of a N-terminal kinase domain similar to those found in other plant protein kinases and a unique C-terminal regulatory domain that acts as an autoinhibitory domain and mediates interactions with CBLs. CBLs bind to a short, conserved region within the C-terminal autoinhibitory domain of CIPKs known as the NAF or FISL motif (Shi *et al.* 1999; Albrecht *et al.* 2001; Guo *et al.* 2001). In addition to modulating the kinase activity of CIPKs, CBLs are thought to be the sole or primary determinants of CBL-CIPK complex localization, therefore they are thought to act as functional modules (Luan 2009; Batistic *et al.* 2011). CBLs are believed to recruit CIPKs, which lack any sort of discernible targeting signals, to these surfaces, possibly in a calcium-dependent manner (Batistič & Kudla 2009; Batistič *et al.* 2010).

Initial functional analysis of the CBL-CIPK network came from the genetic identification of the Salt Overly Sensitive (SOS) pathway. Together, CBL4/SOS3 and CIPK24/SOS2 modulate the activity of the plasma membrane Na^+/H^+ exchanger SOS1. Mutants lacking any component of the Salt Overly Sensitive (SOS) pathway display NaCl-hypersensitive phenotypes (Liu & Zhu 1998; Liu *et al.* 2000; Shi *et al.* 2000). CBL4/SOS3 and CIPK24/SOS2 belong to large protein families containing 10 CBLs and 26 CIPKs in Arabidopsis and similarly sized families in other angiosperms (Kudla *et al.* 1999; Kolukisaoglu *et al.* 2004; Weinl & Kudla 2009). CBL-CIPK complexes have recently been implicated in sodium, potassium, nitrate, and proton transport (Li *et al.* 2006; Xu *et al.* 2006; Ho *et al.* 2009); therefore the CBL-CIPK network is currently thought to be a major regulator of ion homeostasis in angiosperms.

Though CBLs and CIPKs have been discovered among all studied land plants and certain green algal lineages, little is known about the functionality of the CBL-CIPK network outside of angiosperms. As an initial step towards functional analysis of the CBL-CIPK network in an early-diverging land plant, we analyzed the genomic content of CBLs and CIPKs in the model moss *Physcomitrella* and performed bioinformatic analyses of the CBL and CIPK families with an emphasis on relationships among *Physcomitrella* and Arabidopsis CBLs and CIPKs. We classified CBLs according to their phylogeny and N-terminal localization motifs and identified three ancient classes of CBLs. Using yeast two-hybrid analyses, we confirmed interactions among CBLs and CIPKs outside of angiosperms and characterized physical interactions among *Physcomitrella* CBLs and CIPKs. Through phylogenetic analyses, we identified a strongly supported clade that contains all CIPKs identified from green algae and two CIPKs from Arabidopsis and *Physcomitrella*. Using phylogenomic methods, we seek to characterize patterns of expansion of the CBL-CIPK network among land plant lineages to classify CBLs and CIPK in an evolutionarily and functionally meaningful manner to facilitate functional genetic work in early-diverging plants.

Materials & Methods.

Homolog identification, sequence alignment, and bioinformatic analyses.

CBL and CIPK homologs were identified using BLASTp and tBLASTn searches of the Uniprot and the NCBI protein and nucleotide databases, using previously identified CBLs and CIPKs from Arabidopsis as queries. Additional sequences were manually retrieved by annotation from UniProt using the keywords "calcineurin" and "CBL-interacting" (Jain *et al.* 2009). Genomic loci of CBL and CIPK homologs in *Physcomitrella patens* were identified in version 1.6 of the *Physcomitrella* genome, available at http://cosmoss.org (Zimmer *et al.* 2013). All charophyte CBL and CIPK sequences identified were predicted by assembly of homologous expressed sequence tags (ESTs) from transcriptome-level sequencing diverse, representative charophyte genera (Timme & Delwiche 2010; Timme *et al.* 2012). Other new CBL and CIPK protein sequences were predicted from EST sequences in the NCBI non-redundant (nr) nucleotide database identified by tBLASTn searches. Overlapping ESTs from the same taxa were assembled, and ESTs were translated using Geneious R6 (Biomatters), which was also used for all stages of phylogenetic analyses and figure preparation. Predicted CBL and CIPK homologs were verified by manual inspection of domain architecture and pBLAST searches of the NCBI non-redundant (NR) protein database; all protein sequences included in analyses showed expected domain architecture and yielded top BLASTp hits to previously identified CBLs and CIPKs. Protein sequences were aligned using MAFFT (algorithm G-INS-i) and edited and trimmed by eye to remove short, ambiguously aligned regions. Edited alignments were used to generate the phylogenetic trees shown (Katoh *et al.* 2002). Phylogenetic trees were generated from the resulting multiple sequence alignments (MSAs) using PhyML with subtree pruning and regrafting (SPR) + nearest neighbor interchange (NNI) moves and chi-squared-like approximate likelihood ratio test (aLRT) clade support values, which serve as confidence scores much like bootstrap scores. Clades with aLRT scores > .95 were deemed to have strong phylogenetic support (Anisimova & Gascuel 2006; Guindon *et al.* 2009). Specific model parameters are provided in the figure legend for each PhyML analysis presented, however several additional MSAs and evolutionary models and parameters were tested for agreement with conclusions presented here (data not shown). Clades and evolutionary relationships mentioned in the text appeared consistently in independent phylogenetic analyses with different model parameters and MSAs.

Cloning and sequencing of CBLs and CIPKs from the moss Physcomitrella*.*

In order to verify expression and expected splice patterns of CBLs and CIPKs in an early-diverging land plant, we cloned CBLs and CIPKs identified from the model moss *Physcomitrella*. RNA was extracted from protonema and gametophores of *Physcomitrella patens* ssp *patens* ecotype *Gransden 2004* using a CTAB / chloroform method similar to the one described by Chang *et al.*, (1993). The RNA was reverse

transcribed to produce cDNA using Superscript III Reverse Transcriptase (Invitrogen). Primers containing Invitrogen Gateway attB1 (forward primers) and attB2 (reverse primers) recombination sites were designed to amplify the coding sequences (CDSs) of each *Physcomitrella CBL* (*PpCBL*) and *CIPK* (*PpCIPK*) genes (see Table 3.1 for oligonucleotide sequences used in this study). CBL and CIPK transcripts were amplified using Phusion DNA Polymerase (Thermo-Fisher Scientific) following recommended manufacturer protocols on a MJ Research PTC-100 or PTC-200 model thermocycler. PCR products were visualized on a 0.8% agarose gel, and products of the expected sizes were extracted using a QIAquick gel extraction kit (Qiagen) and cloned into the pDONR^{TM}/Zeo vector (Invitrogen) by Gateway BP reaction, following manufacturer recommendations. Samples from three or more clones for each gene were submitted to Elim Biopharmaceuticals, Inc. (Hayward, CA) for DNA sequencing.

Yeast Two-Hybrid Assays.

In order to verify physical interactions among CBLs and CIPKs in a non-angiosperm plant, we cloned the CDS of each full-length *CBL* and *CIPK* transcript identified in *Physcomitrella* and tested interactions among PpCBLs and PpCIPKs in yeast two-hybrid (Y2H) assays using the yeast strain AH109 (Clontech Inc.). This strain is auxotrophic for leucine, tryptophan, histidine, and adenine. The CDSs of *PpCBLs* and *PpCIPKs* were cloned by Gateway LR reaction into yeast two-hybrid gateway-compatible vectors (pGBT9-BS-GW and pGAD-GH-GW) derived from pGBT9-BS and pGAD-GH (Clontech). These vectors were transformed into yeast cells using the G-Biosciences FastYeast Transformation Kit and used to express CBL and CIPK fusions to the DNA-binding domain (BD) and activation domain (AD) of a split transcription factor. We screened CBL-BD fusions (pGBT9-BS-GW constructs) for interactions with CIPK-AD fusion proteins (pGAD-GH-GW constructs) and performed reciprocal screens among CIPK-BD and CBL-AD fusion proteins to verify that the interactions were not vector-dependent. As negative controls, we verified that CBL-BD or CIPK-BD fusion proteins did not interact with the pGAD-GH empty vector (EV).

To perform Y2H screens, co-transformed cells were cultured to mid-log phase in MP Biomedical drop out base (DOB) liquid media lacking leucine and tryptophan (-LT), to ensure retention of vectors containing bait and prey constructs. We then adjusted the cultures to OD_{600} = .05 and divided them into four ten-fold dilutions (OD_{600}= $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$, $5x10^{-5}$). 6 µl droplets of each dilution were plated on agar DOB media (1) lacking leucine and tryptophan (-LT) to serve as a positive control for transformation and loading, (2) lacking leucine, tryptophan, and histidine (-LTH) to test for protein-protein interactions under low stringency, and (3) lacking leucine, tryptophan, histidine, and adenine (-LTHA) to test for interactions under stringent conditions. Cell growth was recorded at 48 hour intervals over the course of six days.

Table 3.1: Oligonucleotides used in this study.

Gene	Forward Primer Name	Forward Primer 5' Gateway Extension (attB1)	Forward Primer Sequence	Reverse Primer Name	Reverse Primer 5' Gateway Extension (attB2)	Reverse Primer Sequence
PpCBL1	PpCBL1-F1g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGGGCTGTTTCCACTCC	PpCBL1-R2g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	TTAGGCACTGCCATCATCC
PpCBL2	PpCBL2-F1g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGGGTTGCTATGGGTCCA	PpCBL2-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	TCAATTATCTTCGACCCGAGAG
PpCBL3	PpCBL3-F1g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGTGGAAGTTATTAGAGCTG	PpCBL3-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	TCAAGTTTCTTCTACTAAGG
PpCBL4	PpCBL4-F4g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGGGGGCTGCGGTG	PpCBL4-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	TCAATCTTTGTCAATTTTGCGAG
PpCIPK1	PpCIPK1-F1g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGAACGGCAACAACAATCGC	PpCIPK1-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	CAATAGTAAATCGCCTCCTTGGC
PpCIPK2	PpCIPK2-F1g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTT	AGTGACCATGACTTCCACC	PpCIPK2-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	AATGCAATTCTAGTAAAGGTACAG
PpCIPK3	PpCIPK3-F1g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGACAACTAAATCAAACATGCC	PpCIPK3-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	TTTCTACTTTTGCTCACTTGTAGG
PpCIPK4	PpCIPK4-F1g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGACTACCGCGACACCAAG	PpCIPK4-R2g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	CTACTTTTGTTCACATGCAGGAAGAG
PpCIPK5	PpCIPK5-F4g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGATGAATTCGAATAGTCTTCGC	PpCIPK5-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	TTACTTCAATGGGGAAACCTTG
PpCIPK6	PpCIPK6-F1g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	ATGAGGAAAGTGGGCAAGTATGAAGTG	PpCIPK6-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	CAGCGGTATATCCTTGCTCCTCATC
PpCIPK7	PpCIPK7-F4g	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC	GAGGAAGTGATGAGGAAGGTG	PpCIPK7-R1g	GGGGACCACTTTGTACAAGAAAGCTGGGTC	CCATACACACACACTTCTATGCTC

Results & Discussion.

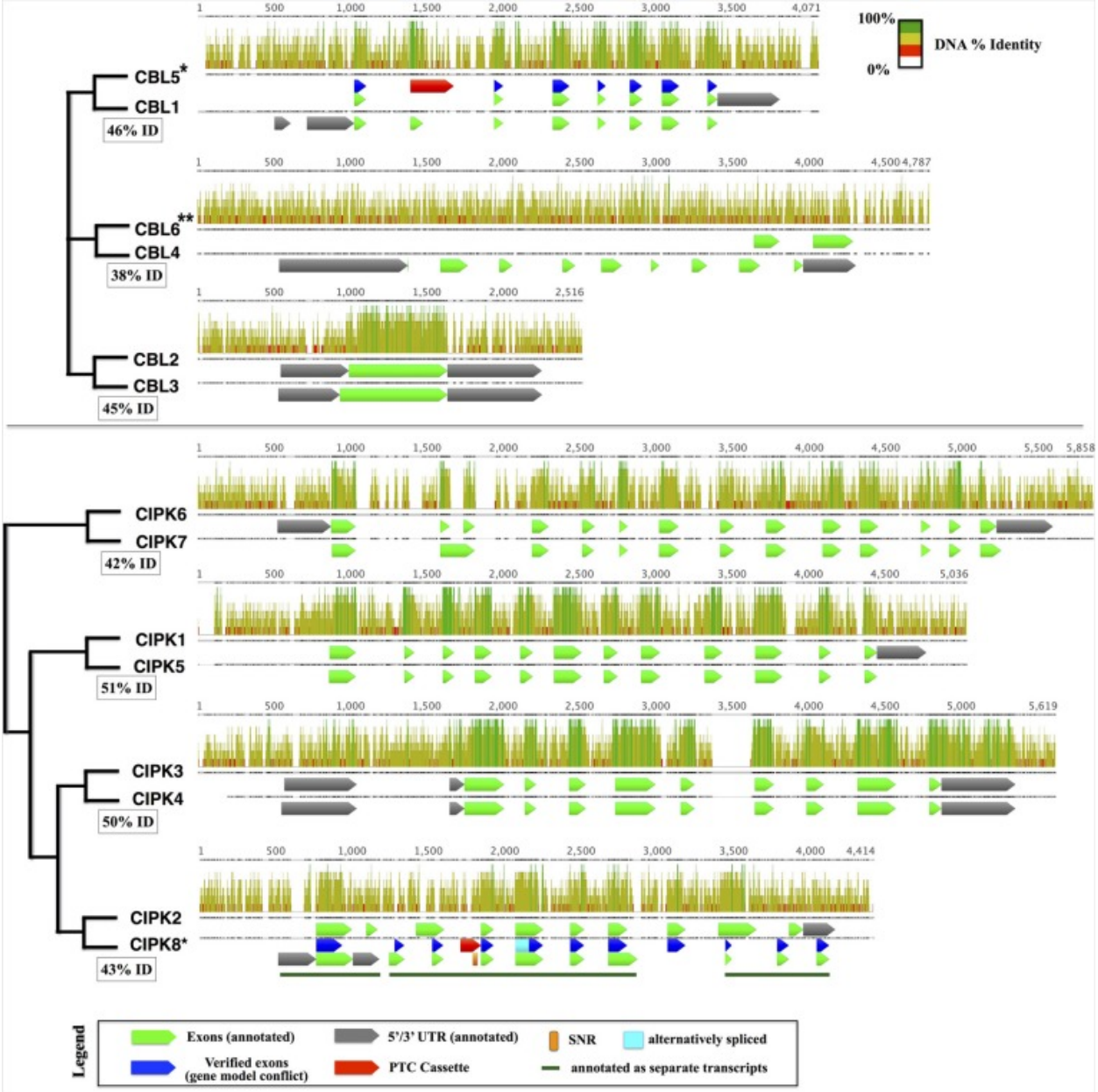
CBL-CIPK network composition in green algae, moss, and other land plants.

CBLs and CIPKs have been previously identified among various land plants and chlorophyte green algae, though other chlorophytes appear to lack CBL-CIPK homologs (Weinl & Kudla 2009). Utilizing recently available transcriptome data, we identified CBL and CIPK homologs from several charophyte green algae species: *Coleochaete orbicularis*, *Klebsormidium flaccidum*, *Chaetospheridium globosum*, *Penium margaritaceum,* and *Chlorokybus atmophyticus*. Interestingly, we identified a single CBL and single CIPK in each of these lineages, with one exception. We could not confidently identify a CIPK homolog from *Chlorokybus*, though this may due to incomplete transcriptome coverage. Additional CBL or CIPK homologs may be present in these taxa but undetected due to incomplete sequencing coverage, or additional homologs may simply not be transcribed at sufficient levels under sampled growth conditions. In agreement with our current understanding of evolutionary relationships among these organisms, charophyte green algae sequences display greater sequence similarity to land plant CBLs and CIPKs than chlorophyte homologs. Although there is no currently available genome sequence for any charophyte, only a single CBL and single CIPK were identified in the complete genome sequence of the chlorophytes *Ostreococcus lucimarinus* and *Bathycoccus prasinos*, consistent with prior findings (Weinl & Kudla 2009). Though it is difficult to make genomic inferences about any charophyte green alga without an available complete genome sequence, our analyses suggest that green algae commonly contain a single CBL-CIPK pair and that the CBL-CIPK network likely predates the split of chlorophyte and charophyte algae.

All CBLs and CIPKs analyzed in this study, including the most divergent homologs identified in algae, show strong conservation of domain architecture and important motifs. At approximately 200 amino acids (AAs) in length, CBLs contain one of a few variations of a localization motif at their N-termini, followed by 4 calcium-binding EF hand domains. The first EF-hand of CBLs is distinctive in that the calcium-binding loop is comprised of 14 rather than 12 AAs, however evidence suggests that it indeed binds calcium ions (Nagae *et al.* 2003). Identified full-length CIPKs are approximately 475 AAs in length and have a conserved domain architecture comprised of a N-terminal kinase domain and a C-terminal autoinhibitory region with a diagnostic NAF domain that mediates interactions with CBLs. One previously identified CIPK from the chlorophyte green alga *Chlorella* (UniProt: C4P7Q5) differs, however, in that it possesses 2 NAF domains in its C-terminus, though the significance and accuracy of the published domain architecture is unknown. Our homology search results corroborate the assertion that CBLs and CIPKs are not found in certain chlorophyte green algae, including the models *Chlamydomonas* and *Volvox* (Weinl & Kudla 2009; Batistic *et al.* 2011). This pattern parallels trends in calcium channel evolution. The *Chlamydomonas* genome encodes several voltage-dependent calcium channels (VDCCs) and transient receptor potential (TRP) channels, which play critical roles in environmental sensing in metazoans, whereas sequenced land plant genomes do not contain discernible

homologs from either family (Wheeler & Brownlee 2008; Verret *et al.* 2010). Like most metazoans, *Chlamydomonas* is motile and, in addition to performing photosynthesis, readily grows heterotrophically. *Chlamydomonas* cells contain an organelle unlike any found in plants, the eyespot, which is involved in the calcium-mediated process of phototaxis (Witman 1993). Based on these observations, it appears that some components of the calcium signaling machinery of certain chlorophyte green algae, such as *Chlamydomonas*, more closely resemble animal signaling networks in some aspects than those of land plants.

Taking advantage of the published genome sequence of the moss *Physcomitrella patens*, we determined the genomic complement of *CBLs* and *CIPKs* in this early-diverging model plant. We identified a total 4 CBL and 7 CIPK predicted protein sequences in *Physcomitrella*, consistent with prior reports (Batistič & Kudla 2009; Weinl & Kudla 2009). One pair of *CBLs* (*PpCBL2*+*3*) and three pairs of *CIPKs* (*PpCIPK1*+*5*, *3*+*4*, and *6*+*7*) showed strikingly high sequence similarities at both the amino acid (73-93% pairwise identity) and genomic level (42-52% pairwise identity). Because of this observation and the inferred whole genome duplication (WGD) estimated to have occurred ~45 million years ago in *Physcomitrella* (Rensing *et al.* 2007), we hypothesized that pairs of *CBLs* and *CIPKs* are products of the recent WGD and that the "unpaired" *CBLs* (*PpCBL1* and *PpCBL4*) and *CIPK* (*PpCIPK2*) may similarly possess cognate loci in the *Physcomitrella* genome. Consistent with this hypothesis, we identified paired loci for each gene and provisionally named these *PpCBL5*, *PpCBL6*, and *PpCIPK8* (Figure 3.1). Although these loci showed relatively low percentage identity to their cognate loci compared to previously detected *CBLs* and *CIPKs*, gene predictions using Augustus (Stanke *et al.* 2004) suggested these loci may encode partial or full-length proteins. Using RT-PCR, we amplified and cloned transcripts from *PpCBL5* and *PpCIPK8*, however we failed to amplify transcripts from the *PpCBL6* locus using several primer pairs validated on genomic DNA (data not shown), despite testing cDNA from different developmental stages (protonema, gametophores, sporophytes). Pairwise alignment of the *PpCBL4* and *PpCBL6* loci revealed a relatively low percentage identity, particularly in *PpCBL4* exonic regions, compared to other "sister" pairs of *CBLs* and *CIPKs*; these observations suggest that *PpCBL6* may be a pseudogene. Sequenced *PpCBL5* and *PpCIPK8* transcripts detected from both gametophyte and sporophyte cDNA were found to contain premature termination cassettes (PTCs) in their spliced forms (see Figure 3.1), which suggests that these transcripts may not be translated, at least under conditions that we tested. *Physcomitrella CIPK8* contains a single nucleotide repeat (SNR), which are known to promote mutations and quickly change in length (Ellegren, 2004), spanning 31 bases in the retained PTC in cloned transcripts, further marking it as an unusual *CIPK*. Interestingly, *PpCBL5* and *PpCIPK8* show obviously stronger conservation in exonic regions (i.e. regions retained in spliced transcripts and that align to their sister gene's CDS) than intronic or regulatory regions (i.e. promoter and terminator). While these aberrant CBL and CIPK loci may simply be in early stages of pseudogenization, the unexpected finding that these loci are transcribed and spliced warrants further investigation into possible functions and may point towards a role for these transcripts



as regulatory RNAs, as shown previously in animals (Korneev et al. 1999; Hirotsune et al. 2003; Tam et al. 2008). Like in *Physcomitrella*, expansion of the CBL-CIPK network in Arabidopsis, previously attributed to segmental duplications (Kolukisaoglu *et al.* 2004), can be traced to known WGD events in light of our current understanding of plant genome evolution (Cui *et al.* 2006). Independent expansion of other gene families in moss and angiosperms has been described, and this can obfuscate direct comparison and functional prediction of genes in widely divergent plants (Cui *et al.* 2006; Bowman *et al.* 2007; Rensing *et al.* 2008; Jiao *et al.* 2011).

Figure 3.1 Pairs of cognate *CBLs* (top) and *CIPKs* (bottom) in the *Physcomitrella* genome aligned using MAFFT. Displayed pairs of CBLs and CIPKs are genomic loci that are reciprocal best BLASTn hits within the genome, and inferred phylogenetic relationships are indicated by cladograms and described in the main text. Pairwise percentage nucleotide (nt) identity for pairs of genomic loci are displayed in boxes. Aligned nucleotides are displayed as bars shaded proportionally to percentage identity, and gapped regions in the alignment are represented by lines. Bar graphs indicate percentage identity (sliding window = 6 nt). Genes with cloned transcripts that do not encode full-length proteins under tested conditions are indicated with an asterisk (*) and genes lacking detectable transcripts are marked with two asterisks (**). In cases where our experimentally inferred gene model did not match the annotation, verified exons (blue), alternatively spliced regions (cyan), and premature termination cassettes (PTCs; red) are shown for comparison. *CIPK8*, which was annotated incorrectly as three separate transcripts, contains a long single nucleotide repeat (SNR) comprised of 31 thymidine (T) residues and described further in the main text. Sequences and associated annotations were extracted from the *Physcomitrella* genome v1.6 starting from 500 nucleotides (nt) upstream of the annotated 5′ UTR (750 nt upstream CDS for genes lacking 5′ UTR annotations) to 250 nt downstream of the annotated 5′ UTR (500 nt downstream the CDS) were extracted. Pairwise loci were aligned using MAFFT. Although *PpCBL6* has annotated exons, there is no experimental evidence that any part of this locus in transcribed.

Phylogenomic analysis of CBLs reveals conservation of three unique N-terminal motifs.

Phylogenomic methods extend the ability to determine relationships among distant homologs, facilitate functional prediction, and provide a framework for discovery of key features by identifying conserved regions of proteins (Eisen & Wu 2002; Sjölander 2004). Using maximum likelihood (ML) methods, we reconstructed the phylogeny of the CBL family in green lineages. Consistent with our hypothesis that land plant CBLs and CIPKs expanded from a simple module present in their common ancestor with algae, green algal CBLs cluster closely to one another with high confidence scores in phylogenetic analyses. Although algal CBLs do not consistently cluster with any particular clade of CBLs from land plants, they commonly show moderate phylogenetic affinity for a clade containing Arabidopsis CBL1 and CBL9 (Figure 3.2), which play important roles in potassium nutrition through regulation of the AKT1 channel. Like Arabidopsis CBL1 and CBL9, green algal CBLs feature the dual-lipid modification motif MGCXXS/T or obvious relicts of this motif. Due to the retention of this motif among many embryophyte and green algal CBLs and the results of our phylogenetic analysis, we hypothesize that the dual-lipid modification motif is the ancestral localization mechanism of CBLs. This hypothesis is strengthened by the observation that distantly homologous neuronal calcium sensor (NCS) proteins feature a similar N-terminal motif (MGXXXS) that lacks the conserved cysteine residue but does trigger N-myristoylation of the conserved glycine residue (Li *et al.* 2011). We designate homologs with the dual lipid modification motif as Type I CBLs (Figure 3.3). Consistent

with the hypothesis that ancestral CBLs most closely resembled modern Type I homologs and gave rise to other types of CBLs, Type I CBLs are paraphyletic with respect to other CBLs. Arabidopsis CBLs containing the Type I dual lipid modification motif have been shown to localize to the plasma membrane (D'Angelo *et al.* 2006; Cheong *et al.* 2007; Batistič *et al.* 2008). Mutational analyses using FP-fusions indicate that both N-myristoylation and S-acylation are required to target proteins to the plasma membrane, whereas either modification on its own results in endomembrane localization (Batistič *et al.* 2008). Although subcellular localization has not been investigated in early diverging plants or green algae, we speculate that the ancestral CBL-CIPK module may have participated in the regulation of integral membrane proteins at the plasma membrane, given the observed evolutionary trends and our understanding of CBL-CIPK function and biochemistry in Arabidopsis.

Phylogenetic analyses revealed a strongly supported (aLRT = 1.0) clade that contains *Physcomitrella* CBL2 and CBL3 and Arabidopsis CBL2, CBL3, CBL6, and CBL7 (Figure 3.4). *Physcomitrella CBL2* and *CBL3* encode proteins that are 76% identical, and both genes lack introns, unlike other *CBLs* from Arabidopsis or *Physcomitrella*. The clade also contains homologs from other non-angiosperms, including three CBLs from the lycophyte *Selaginella moellendorffii*. Like Arabidopsis,

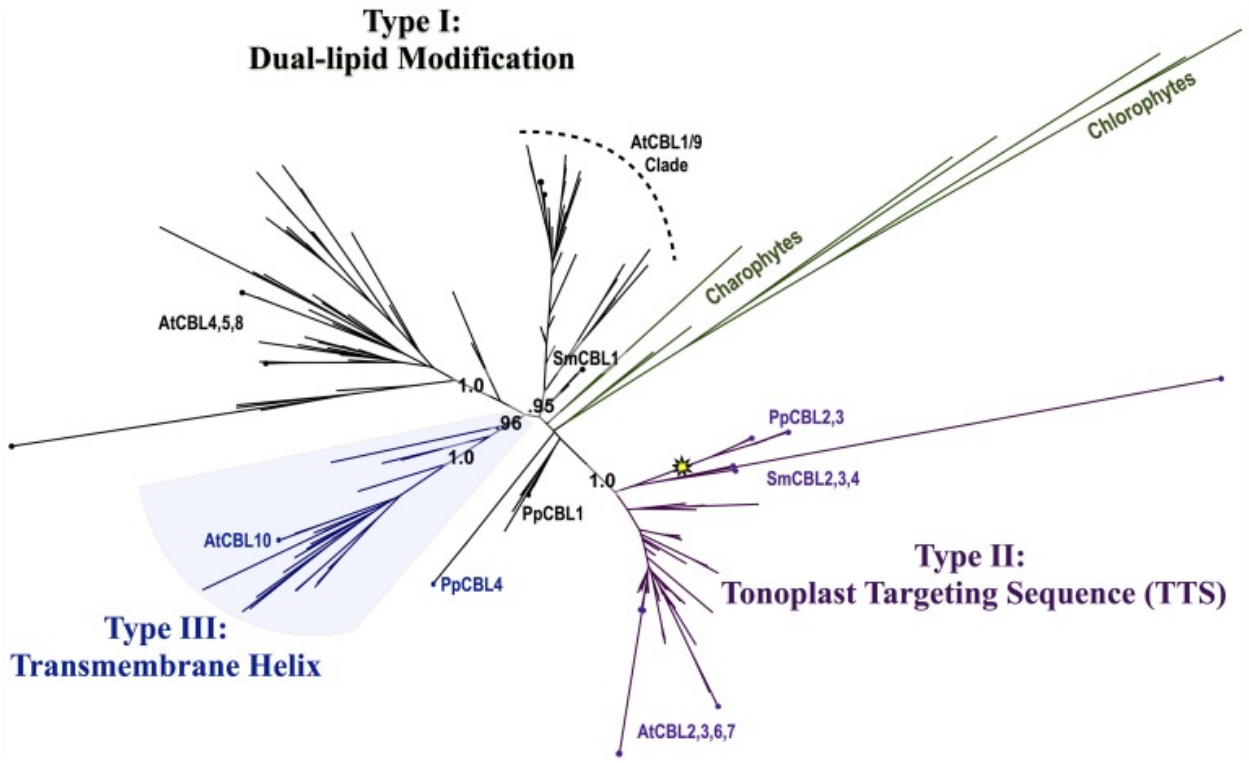
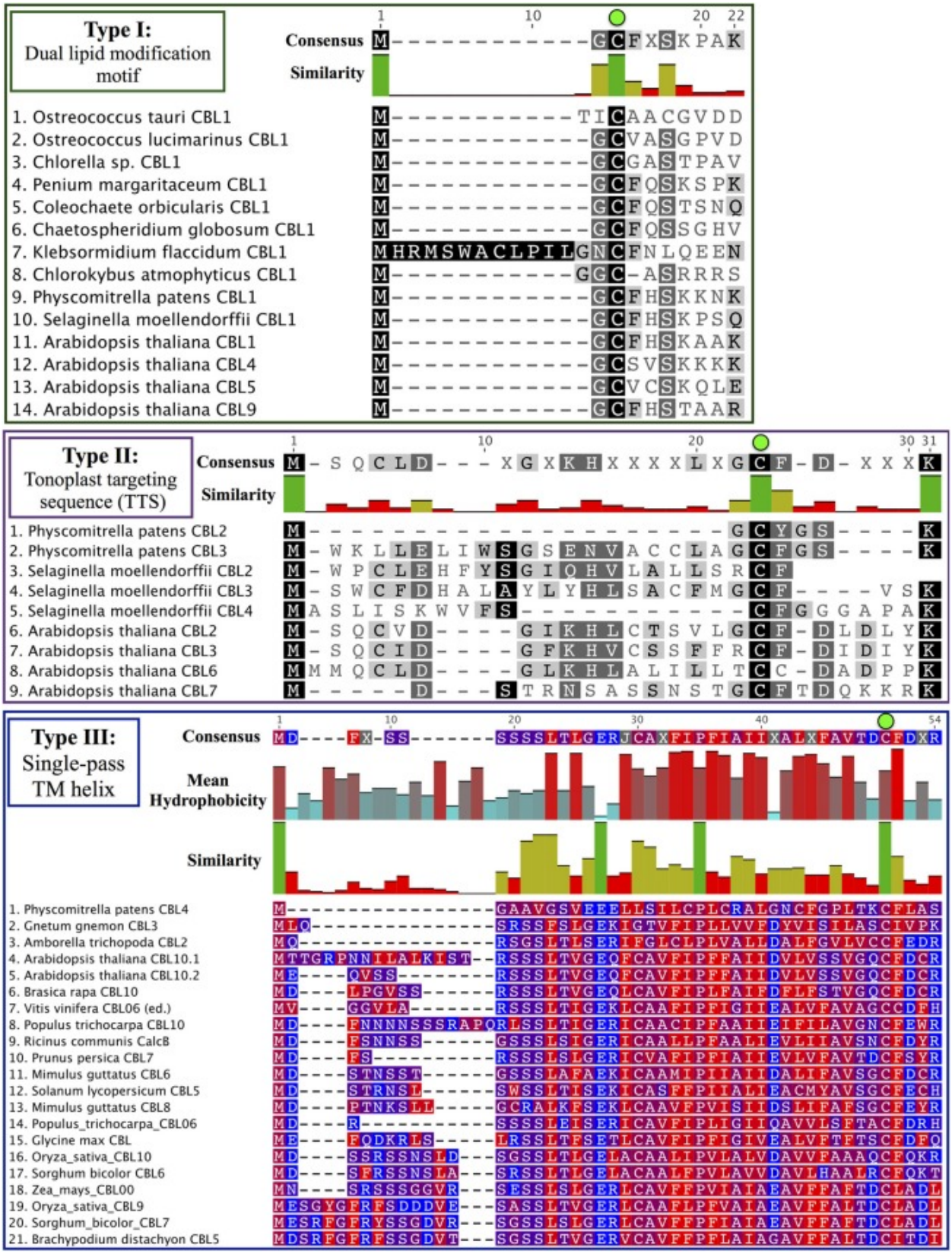


Figure 3.2: Maximum Likelihood (ML) phylogenetic tree derived from multiple sequence alignment (MSA) of all CBL amino acid (AA) sequences analyzed in this study. Chlorophyte and charophyte green algal CBL and CIPKs are highlighted in green, and clades containing Type I–III CBLs identified in this study are annotated. The yellow star indicates an inferred intron loss event**.**

Selaginella CBLs in this clade contain multiple introns and exhibit a conserved exon-intron structure, leading us to infer there was a likely reverse transcription event in the *Physcomitrella* lineage not shared with the lineage leading to lycophytes and angiosperms. Experimental work is needed to determine functional consequences of intron loss in *Physcomitrella CBL2* and *CBL3*, however the effects and mechanisms of reverse transcription-mediated intron loss events and other means of intron loss are discussed elsewhere (Jeffares *et al.* 2006; Filichkin *et al.* 2010). Based on high sequence similarity, shared intron loss, and strong phylogenetic evidence, we infer that *PpCBL2* and *PpCBL3* are products of a lineage-specific gene duplication, likely the results of a recent WGD (Rensing *et al.* 2007, 2013). Both genes are orthologous to the four Arabidopsis *CBLs* contained in this clade. Arabidopsis *CBL2* and *CBL3* are also recent duplicates, as evidenced by their phylogenetic placement and very high sequence similarity (~92% AA identity) throughout their entire lengths. *CBL3* and *CBL7* are tandem duplicates, although CBL7 is disparate from other Arabidopsis CBLs and contains a deletion in its N-terminus between a degenerate dual-lipid modification motif and its first EF hand (Batistič & Kudla 2009). Arabidopsis CBL6, which features an unusual first EF hand relative to other CBLs, is more distantly related to the three other AtCBLs in this clade and forms a clade with orthologous CBLs from other eudicots. Arabidopsis CBL2, CBL3, and CBL6 have been reported to localize to the tonoplast (Batistič & Kudla 2009). In the case of CBL2 and CBL3, it has been rigorously shown that an N-terminal motif known as the tonoplast targeting sequence (TTS) mediates its subcellular localization (Tang *et al.* 2012). The TTSs of Arabidopsis CBL2 and CBL3, with the consensus motif MSQCXDGXKHXCXSXXXCF, span 19 AA; and the last three positions of the motif overlap with positions 2-4 in the dual lipid modification motif of Type I CBLs (i.e. MGCXXS/T), sharing a conserved cysteine residue found in all CBLs analyzed (see Figure 3.3). This 19-AA fragment from either Arabidopsis CBL2 or CBL3 is necessary and sufficient for targeting of FP fusions to the tonoplast in Arabidopsis mesophyll cells (Tang *et al.* 2012), and strong sequence similarity suggests that CBL6 shares this targeting mechanism (Fig. 3.3 *middle*).

Figure 3.3: CBL N-terminal localization motifs can be classified into three ancient types. Consensus sequences are provided above each MSA, and degree of conservation is indicated by bar graph and shading. Note the strictly conserved cysteine residues (green dots) in all three types of CBLs. (Top) Type I CBLs harbor a dual-lipid modification motif (MGCXXS/T) that triggers N-myristoylation of the glycine residue and S-acylation of the cysteine residue. Most green algal CBLs identified to date are Type I CBLs or appear to retain signatures of the dual-lipid modification motif. (Middle) Type II CBLs are characterized by a N-terminal extension called the TTS that is found in nearly all CBLs contained in the Type II clade. Phylogenetic evidence suggests that PpCBL2 has lost its TTS through a mechanism such as gene conversion. (Bottom) Type III CBLs feature a long N-terminal extension that is predicted to constitute a transmembrane helix. Residues are colored according to hydrophobicity (red) or hydrophilicity (blue), and mean hydrophobicity and similarity are indicated by bar graphs. Although PpCBL4 does not cluster with seed plant CBLs that share a similar N-terminal extension, we propose that it is targeted in a similar manner to other Type III CBLs based on sequence analysis of its N-terminal extension.


Type I:
Dual lipid modification motif
Consensus
Similarity
1. Ostreococcus tauri CBL1
2. Ostreococcus lucimarinus CBL1
3. Chlorella sp. CBL1
4. Penium margaritaceum CBL1
5. Coleochaete orbicularis CBL1
6. Chaetospheridium globosum CBL1
7. Klebsormidium flaccidum CBL1
8. Chlorokybus atmophyticus CBL1
9. Physcomitrella patens CBL1
10. Selaginella moellendorffii CBL1
11. Arabidopsis thaliana CBL1
12. Arabidopsis thaliana CBL4
13. Arabidopsis thaliana CBL5
14. Arabidopsis thaliana CBL9
Type II:
Tonoplast targeting sequence (TTS)
Consensus
Similarity
1. Physcomitrella patens CBL2
2. Physcomitrella patens CBL3
3. Selaginella moellendorffii CBL2
4. Selaginella moellendorffii CBL3
5. Selaginella moellendorffii CBL4
6. Arabidopsis thaliana CBL2
7. Arabidopsis thaliana CBL3
8. Arabidopsis thaliana CBL6
9. Arabidopsis thaliana CBL7
Type III:
Single-pass TM helix
Consensus
Mean Hydrophobicity
Similarity
1. Physcomitrella patens CBL4
2. Gnetum gnemon CBL3
3. Amborella trichopoda CBL2
4. Arabidopsis thaliana CBL10.1
5. Arabidopsis thaliana CBL10.2
6. Brasica rapa CBL10
7. Vitis vinifera CBL06 (ed.)
8. Populus trichocarpa CBL10
9. Ricinus communis Calc8
10. Prunus persica CBL7
11. Mimulus guttatus CBL6
12. Solanum lycopersicum CBL5
13. Mimulus guttatus CBL8
14. Populus_trichocarpa_CBL06
15. Glycine max CBL
16. Oryza_sativa_CBL10
17. Sorghum bicolor CBL6
18. Zea_mays_CBL00
19. Oryza_sativa_CBL9
20. Sorghum_bicolor_CBL7
21. Brachypodium distachyon CBL5

CBL7 is reported to show a diffuse nuclear and cytosolic localization based on the analysis of fluorescent fusion proteins (Batistič *et al.* 2008), however we are unaware of any rigorous attempts to determine its subcellular localization. Therefore, it appears that tonoplast localization is a generally conserved feature among angiosperm CBLs in this clade. We identified a TTS-like motif in all three *Selaginella* CBLs in this clade and in PpCBL3. Unlike PpCBL3, PpCBL2 does not contain an extended N-terminus and instead contains the Type I dual lipid modification motif. Given these trends, we posit that the TTS is a synapomorphy of this clade and that PpCBL2 lost its TTS via deletion or partial gene conversion, as described elsewhere (Jeffares *et al.* 2006). Based on strong phylogenetic support and TTS motif conservation, we designate homologs contained in this clade as Type II CBLs.

Phylogenetic analyses also identified a strongly supported clade that contains Arabidopsis CBL10, the only Arabidopsis CBL predicted to contain a transmembrane (TM) helix for membrane association (Figure 3.5). This clade contains orthologs from all studied angiosperms and gymnosperms, indicating this clade is conserved among seed plants; and all members of this clade with full-length sequences exhibit a predicted N-terminal transmembrane helix. Like members of the AtCBL10 clade, *Physcomitrella* CBL4 contains an extended N-terminus, which we posited may form a transmembrane helix (Fig. 3.3 *bottom*). Various TM topology prediction methods disagree on whether AtCBL10 or PpCBL4 contain a predicted TM helix (data not shown), however visual inspection of hydrophobicity and patterns of conservation in MSAs suggests that both AtCBL10 and PpCBL4 contain N-terminal TM helices. The presence of this TM helix raises the possibility that PpCBL4 may be an AtCBL10 ortholog, however our phylogenetic data neither favor nor disfavor this hypothesis. More thorough coverage of sequence data from early-diverging plants is likely required to test this possibility and determine whether Type III CBLs are monophyletic or not. The Arabidopsis *CBL10* transcript is reportedly processed into mRNAs that encode proteins with two distinct N-termini, though both share the same TM helical region. Alternative splicing is mediated by a unique 8th intron (other rice and Arabidopsis CBLs contain 6 or 7 introns) towards the 5'pend of the transcript. Both *Physcomitrella CBL4* and Arabidopsis *CBL10* share a very similar exon-intron structure (data not shown), though we did not find evidence of alternative splicing in *PpCBL4*.

Figure 3.4: Close-up of Type II CBL clade from global ML tree. Confidence scores (aLRT) are shown for each clade, and the yellow star denotes an inferred intron loss event. Some clades are unlabeled or collapsed for clarity. Type II CBLs are distinguished by the presence of an N-terminal tonoplast targeting sequence (TTS), however certain members (asterisks) of the Type II clade have lost or degenerate TTSs. Arabidopsis CBLs in this clade (green dots) contain the TTS or a degenerate form of it; whereas the two *Physcomitrella* CBLs (yellow dots) in this clade sharply differ in that CBL2 has a Type I dual-lipid modification motif and CBL3 has a TTS. Presence of the TTS in *Selaginella* homologs within the clade suggests that CBL2 has lost its TTS, through a mechanism such as gene conversion, for example.


Funaria_hygrometrica_CBL03_ctg8
Funaria_hygrometrica_CBL02_ctg101
Physcomitrella_patens_CBL02_C4P7X5
Pohlia_nutans_CBL02_ctg1
Ceratodon_purpureus_CBL03_ctg9515
Funaria_hygrometrica_CBL05_ctg7
Physcomitrella_patens_CBL03_C4P7X4
Ceratodon_purpureus_CBL02_ctg17665
Pohlia_nutans_CBL01_GACA01010238.1
Selaginella_moellendorffii_CBL02_C4P7X7
Selaginella_moellendorffii_CBL03_C4P7X8
Selaginella_moellendorffii_CBL04_C4P7X9
Adiantum_capillus-veneris_CBL02_|gb|BP916317.1...
Pteridium_aquilinum_CBL01_ctg3640
Pteridium_aquilinum_CBL03_ctg5
Ceratopteris_richardii_CBL01_|gb|BE643190.1|
Adiantum_capillus-veneris_CBL06_|gb|DK949513.1...
Pteridium_aquilinum_CBL05_ctg3
Pinus
Picea
Cryptomeria_japonica_CBL01_gb|BY892361.1|BY878...
Gnetum_gnemon_CBL01_|gb|EX945622.1|EX948559.1|
Cryptomeria_japonica_CBL06_|gb|BY895641.1|
Pinus_banksiana_CBL02_gb|GW760970.1|GW773193.1...
Ginkgo_biloba_CBL01_|gb|EX939936.1|DR073496.1|...
Gnetum_gnemon_CBL02_|gb|EX945295.1|DN954751.1|
Cycas_rumphii_CBL03_|gb|EX926078.1|
Zamia_vasquezii_CBL01_|gb|FD775526.1|
Picea_glauca_CBL04_gb|EX380755.1|EX389721.1|CO...
Pinus_contorta_CBL02_gb|GT257254.1|GT251544.1|...
Pinus_contorta_CBL04_gb|GT234406.1|GT234743.1|
Cryptomeria_japonica_CBL07_|gb|BY877934.1|
Mimulus_guttatus_CBL03_mgf009075m
Medicago_truncatula_Unchar_B7FN44
Amborella_trichopoda_CBL01_gb|FD426708.1|FD442...
Gossypium_hirsutum_CBL02_A8TWZ6
Gossypium_hirsutum_CBL03_A8TWY9
Prunus_persica_CBL06_ppa011040m
Ricinus_communis_CalcB_B9T1Q9
Vitis_vinifera_CBL02_A5BYS8
Cryptomeria_japonica_CBL04_|gb|BY905435.1|BY89...
Arabidopsis_thaliana_CBL02_Q8LAS7
Brassica_rapa_CBL02_AC189378(EXCV544920)
Arabidopsis_thaliana_CBL03_Q8LEM7
Arabidopsis_thaliana_CBL07_Q9SUA6
Populus_trichocarpa_CBL2-2_A4ZKI4
Populus_trichocarpa_CBL2-1_A4ZKI3
Zingiber_officinale_CBL01_gi|87076007
Musa_acuminata_CBL1-1_gi|182665920
Musa_acuminata_CBL1-2_gi|182665988
Oryza_sativa_CBL06_Q3HRP1
Brachypodium_distachyon_CBL01_Bradi1g28160.1
Brachypodium_distachyon_CBL10_Bradi4g41910.1
Sorghum_bicolor_CBL02_C4P7Y5
Zea_mays_CBL00_B6TXZ0
Lilium_longiflorum_CBL01_gi|73944713
Oryza_sativa_CBL03_Q75LU8
Zea_mays_CBL03_B6TBI3
Brachypodium_distachyon_CBL09_Bradi4g02740.1
Oryza_sativa_CBL02_Q3HRP5
Sorghum_bicolor_CBL03_C4P7Y6
Zea_mays_CBL00_B6T8N9
Vitis_vinifera_CBL03_A5AHA7
Mimulus_guttatus_CBL02_mgf011572m
Brassica_rapa_CBL06_AC189608
Arabidopsis_thaliana_CBL06_Q9C5P6
Populus_trichocarpa_CBL03_A4ZKJ1
Ricinus_communis_CalcB_B9SGB5
Glycine_max_CBL01_Gm0159x00019
0.3 substitutions per site

The typically short length and strong structural conservation of EF hand proteins like CBLs can complicate phylogenetic reconstruction, as relatively few substitutions can significantly influence results. Due to biophysical constraints, EF hand domains typically exhibit strong sequence conservation at positions that coordinate calcium ion binding. However, variation seen among EF hands of CBLs are predicted to have widely differing affinities for calcium ions, thereby facilitating functional diversity at the level of calcium binding. The 4th EF hand (EF4) of *Physcomitrella* CBL4 is unusual in that it contains nonpolar residues at two of the positions that coordinate calcium ion binding, rather than negatively charged residues as seen in virtually all other EF hands. Therefore, it appears likely that it does not bind calcium. Indeed, studies of other calcium signaling pathways have underscored the plasticity of signaling components during evolution.

The model yeast *Saccharomyces cerevisiae* contains a single-copy gene encoding a calmodulin (CaM), a widely studied type of calcium sensor in eukaryotes. This gene, *CMD1*, is indispensable for survival of the cell. Surprisingly, molecular genetic analysis suggests the CaM's ability to bind calcium ions is dispensable for its most vital functions, and its fourth EF hand is unable to bind calcium (Cyert, 2001). Plants contain a suite of typical CaMs and widely divergent CaM homologs, some of which either lack the ability to bind calcium ions or coordinate them in an unusual manner (McCormack & Braam 2003). Further work is needed to clarify the capacity and affinity of identified CBLs for calcium binding, particularly among non-angiosperm CBLs.

There has been some debate as to the localization of Arabidopsis CBL10; various reports indicate localization to the tonoplast (Kim *et al.* 2007; Batistič *et al.* 2010) or plasma membrane (Quan *et al.* 2007; Ren *et al.* 2013). Although the multiple isoforms of CBL10 may account for different localization patterns, Arabidopsis CBL10 is most strongly expressed in shoots and is suggested to participate in the regulation of a NHX-family, Na^+/H^+ exchanger believed to function in the sequestration of sodium ions within the vacuole. A model has emerged wherein CBL10 plays a regulatory role in the SOS pathway akin to that of CBL4/SOS3 (Kim *et al.* 2007). In root hair and cortical cells, the Type I Arabidopsis CBL4 forms a complex with CIPK24, and together they regulate the activity of the plasmalemma-localized Na^+/H^+ exchanger SOS1 (syn. NHX7) and facilitate the extrusion of sodium ions from the plant. In shoot mesophyll cells, CBL10 complexes with CIPK24, and together they putatively regulate the activity of an unidentified tonoplast-localized Na^+/H^+ exchanger and facilitate sequestration of sodium ions in the vacuole. A recent publication proposes a role for CBL10 in the regulation of the plasmalemma-localized potassium channel AKT1 (Ren *et al.* 2013), which has been rigorously shown to be subject to regulation by CBL1 and CBL9 acting in concert with CIPK23 (Li *et al.* 2006; Xu *et al.* 2006). Our phylogenetic results indicate that the single-pass N-terminal TM helix is a synapomorphy of the AtCBL10 clade. *Physcomitrella* CBL4 likewise contains a N-terminal TM helix and may be orthologous, therefore we designated these homologs Type III CBLs.

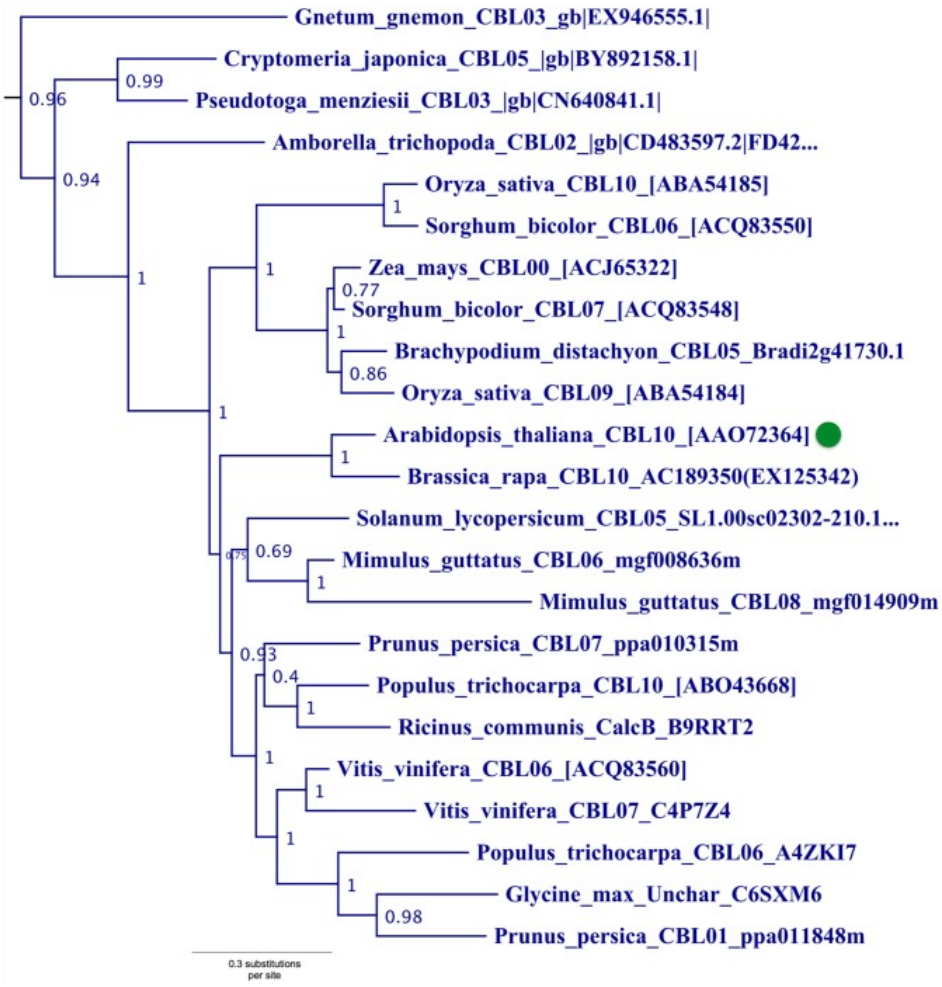


Figure 3.5: Close-up of Type III CBL clade from ML tree shown in Figure 3.2. Confidence scores (aLRT) are shown for each clade. Arabidopsis CBL10 (green dot) and orthologs in angiosperms and gymnosperms that share the presence of a single N-terminal transmembrane helix (see Figure 3.3) form a well-supported clade (aLRT = 0.96). *Physcomitrella* CBL4, which also appears to contain a N-terminal transmembrane helix, does not phylogenetically cluster with this clade or other angiosperm homologs, possibly due to sparse taxon sampling among bryophytes.

Different membranes of the eukaryotic cell have distinct phospholipid profiles, which can serve as a basis for subcellular targeting. Moreover, each particular membrane is commonly composed of distinct microenvironments with unique lipid and protein populations. Together, proteins and lipids are thought to form functional modules in cellular membranes, with membrane-targeted kinases recognized as common regulatory modules (Engelman 2005). For these reasons, we expect that CBL-CIPK complexes are likely targeted not only to specific membranes but to precise sites within membranes where they interact and function with molecular partners (Bhatnagar & Gordon 1997; Levental *et al.* 2010). Elevation of free calcium in the cytosol is localized and transient, partly due to effects of Ca^{++}-ATPases and Ca^{++}/H^{+} antiporters and proteins that act as buffers. Because calcium signatures occur locally, calcium sensors must operate in close proximity to the channels responsible for calcium elevation (Fogelson & Zucker 1985; Gilroy *et al.* 1993; Roberts 1994; Clapham 2007). In light of this, we interpret the conservation of CBL localization motifs among distantly related plants as a likely consequence of constraints on CBL-CIPK subcellular localization.

Although several studies have examined CBL localization, it remains unclear whether CBLs display a predominantly static or dynamic localization at protein maturity. Our analyses demonstrate that the cysteine residue occupying the third position in the Type I motif (MGCXXS/T) is perfectly conserved among CBLs from widely divergent organisms and paralogous clades. In Type I and Type II CBLs, this residue has been shown to be S-acylated, and the modification is required for known protein functions (Batistič *et al.* 2008, 2012; Tang *et al.* 2012). Based on its striking conservation, we predict that S-acylation of this conserved residue is a shared among CBLs, at least under certain conditions. It is well established that S-acylation is a reversible post-translational modification and that it can strongly impact protein localization and can be critical for protein function (Bijlmakers & Marsh 2003; Hemsley & Grierson 2008). Prior research has pointed towards a role for S-acylation in fine-level targeting of proteins to specific membrane microenvironments (Bhatnagar & Gordon 1997; Mumby 1997; Dunphy & Linder 1998; Levental *et al.* 2010). We predict that N-terminal S-acylation at this conserved residue functions, at least in part, as a mechanism for precise and dynamic localization of CBLs.

Conservation of the NAF motif and CBL-CIPK interactions in Physcomitrella.

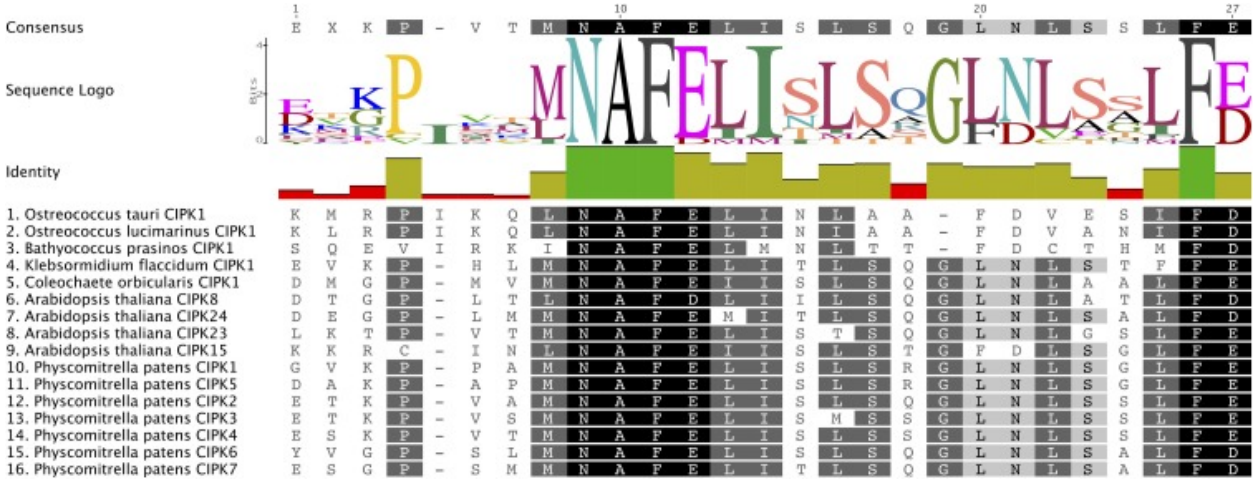
The CBL-CIPK network is mediated by a conserved CBL-interacting domain (also known as the NAF or FISL motif) in CIPKs. Our MSA of the CIPK family indicates that the NAF domain is strongly conserved, with many identical residues, among algal CIPKs and all CIPKs from Arabidopsis and *Physcomitrella* (Figure 3.6). This observation is consistent with our prediction that CBLs and CIPKs from green algae and early diverging embryophytes function together as a module. To confirm our presumption that *Physcomitrella* CBLs and CIPKs physically interact with each other and lend support to our interpretation of these protein families as functionally connected

in early-diverging plants, we performed Y2H screening and characterized physical interactions between full-length PpCBLs and PpCIPKs in yeast cells.

Consistent with our expectations, CBLs and CIPKs from *Physcomitrella* showed physical interactions in yeast cells. All combinations of PpCBL and PpCIPK fusion proteins showed physical interactions in yeast (Figure 3.7), but specific CBL-CIPK combinations showed very strong interactions with select partners, consistent with the hypothesis that particular CBLs show preferential interactions with cognizant CIPKs (Figure 3.8). We observed that "sister" CIPKs displayed overlapping, though not identical, interaction profiles with their most closely related homolog. CIPK1 and CIPK5 interact moderately with CBL2 and CBL4 and weakly with CBL1 and CBL3. CIPK3 and CIPK4 interact weakly with CBL3 but moderately to strongly with CBL1, 2, and 4. CIPK6 and CIPK7 interact strongly with CBL4 and weakly to moderately with CBL1, 2, and 3. We observed only weak interactions between CIPK2, which lacks a "sister" CIPK, and any CBL, despite conservation of its NAF domain and phylogenetic proximity to the highly interactive CIPK3 and CIPK4.

Among the CBLs, CBL4 shows the highest number of strong connections to CIPKs, and it interacts very strongly with CIPK6 and CIPK7, members of the green algal clade of CIPKs. CBL1, a Type I CBL without clear phylogenetic affinities to angiosperm CBLs, most strongly interacts with CIPK4 and shows very weak interactions with CIPK1 and CIPK5. CBL3 shows clearly weaker interactions with CIPKs than its close paralog CBL2, although their interaction profiles are similar. CBL2 and CBL3 both interact most strongly with CIPK3, CIPK4, and CIPK6. These data support the model of a highly interconnected signaling network; however, interaction patterns may differ significantly in moss cells due to differences in post-translational modifications, subcellular localization, expression, and other factors. Nonetheless, these results provide a guide

Figure 3.6: The CBL-interacting or NAF motif is conserved in green algal and moss CIPKs. Consensus amino acid sequence of this motif and degree conservation, illustrated by bar graph, are provided above the MSA, which is shaded based on the degree of AA conservation. Strong conservation of this motif, responsible for CIPK interactions with CBLs, suggests that CBLs and CIPKs identified from green algae and early-diverging land plants constitute a functionally linked network.



for genetic analyses in moss and lend confidence to the interpretation that CBLs and CIPKs are functionally linked in early-diverging plants and constitute an ancient signaling network.

Phylogenomic identification of the the Ancestral or "green algal-type" clade of CIPKs.

Phylogenomic analyses of the CIPK family were pursued, as described for CBLs, to decipher evolutionary patterns to facilitate identification of functionally meaningful groups, which would be expected to show conservation across diverse land plants. Our phylogenomic analyses of CIPKs (Figure 3.9) indicated most Arabidopsis CIPKs (18 of 26) are contained within an "intronless" clade (although *CIPK16* contains a single intron that is inferred to be from an intron-gain event), consistent with prior analyses by Kolukisaoglu *et al.* (2004). We used conifer protein sequences from this clade as queries for tBLASTn searches of *Picea* chromosomal sequences (available at http://congenie.org) and did not identify introns in expected locations for intron-containing CIPKs (data not shown). Based on these observations, we posit that a reverse transcription event occurred before the split of gymnosperms and angiosperms and is a conserved feature of this clade.

All *Physcomitrella CIPKs* contain multiple introns, and none cluster with the intronless clade. *Physcomitrella* CIPK1 - CIPK5 share high sequence similarity (83-93% pairwise); and in our analyses, they were placed with strong confidence in a clade with homologs from other mosses, indicating they are paralogs in respect to their closest seed plant homologs. This clade of moss homologs is likely orthologous (aLRT = 0.97) to three clades of CIPKs conserved across seed plants: the aforementioned intronless clade, a clade containing AtCIPK21, and a clade containing AtCIPK1 and AtCIPK17. Arabidopsis CIPK3+CIPK26, CIPK9, and CIPK23 each represent strongly supported (aLRT = 1.0) clades that cluster with one another and contain homologs in fully sequenced angiosperm genomes and, at least for the CIPK3+CIPK26 and CIPK23 clades, in gymnosperms. Whereas CIPK9 and CIPK23 regulate potassium transport and function in root and shoot tissues (Pandey *et al.* 2007; Cheong *et al.* 2007), CIPK3 has been implicated in abscisic acid (ABA)-dependent regulation of seed germination (Pandey *et al.* 2008), therefore homologs from seed plants as distantly related as gymnosperms might conceivably have a conserved regulatory role in seeds, given their strong conservation.

Physcomitrella CIPK6 and CIPK7 belong to a clade that contains Arabidopsis CIPK8 and CIPK24 and, importantly, contains all green algal CIPKs identified with high confidence (aLRT = 1.0). Although *Physcomitrella* and Arabidopsis each contain two homologs in this clade, *Physcomitrella* CIPK6 and CIPK7 (72% AA pairwise identity) are the products of a gene duplication that occurred after the split between mosses and the lineage leading to vascular plants (Figure 3.10). In contrast, Arabidopsis CIPK8 and CIPK24 (60% pairwise identity) each represent a separate, strongly supported clade with orthologs in other angiosperms, implying that they derive from duplications that occurred during seed plant (most likely angiosperm) diversification.

Figure 3.7: Yeast two-hybrid interactions among Physcomitrella CBL-CIPK pairs. Each CBL and CIPK was fused to activation domain (AD) or DNA-binding domain (BD) of a split transcription factor and screened for interactions Interaction strength was inferred by serial growth dilutions on selective media lacking one (-LTH) or two (-LTHA) auxotrophic markers.

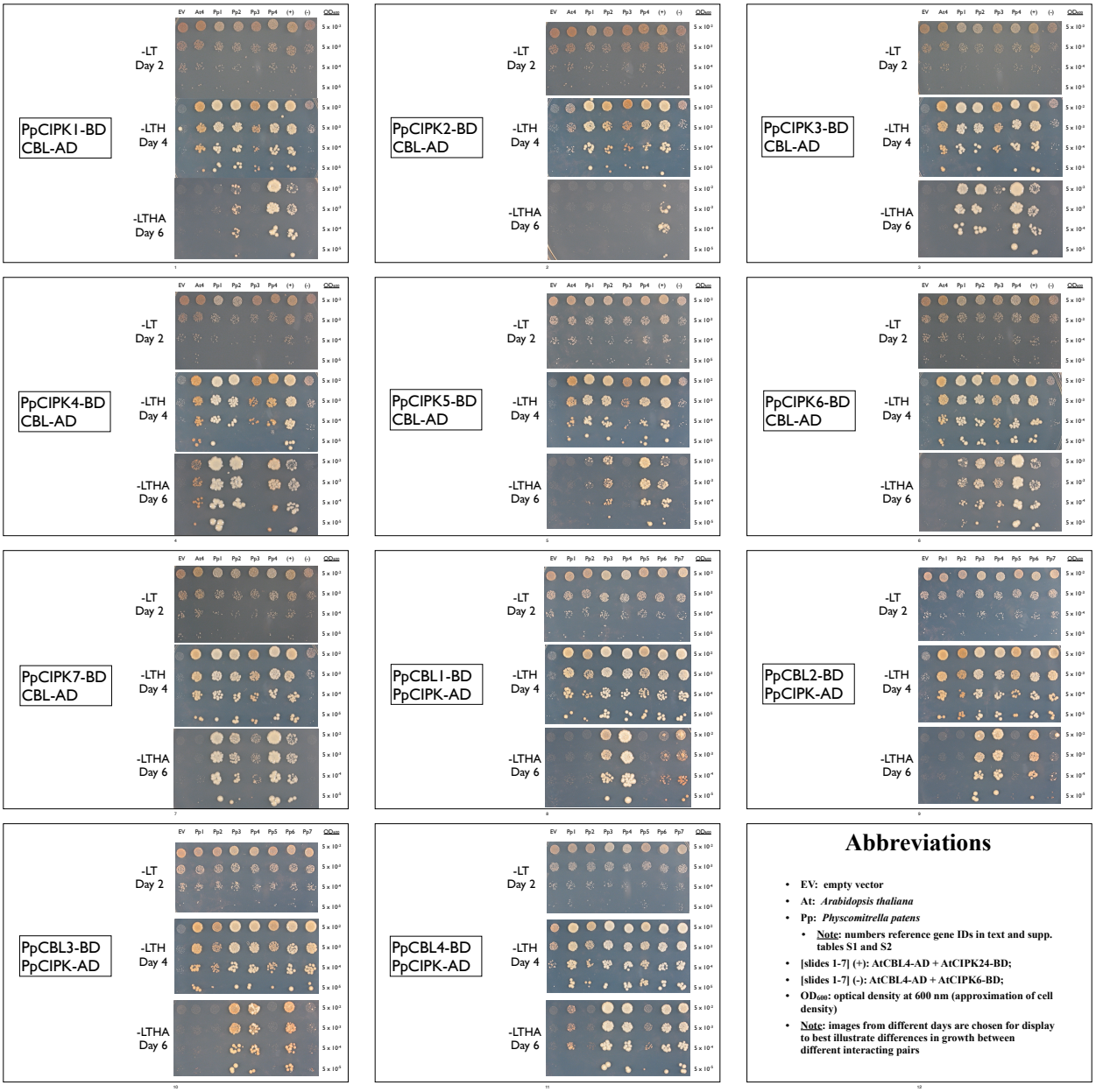
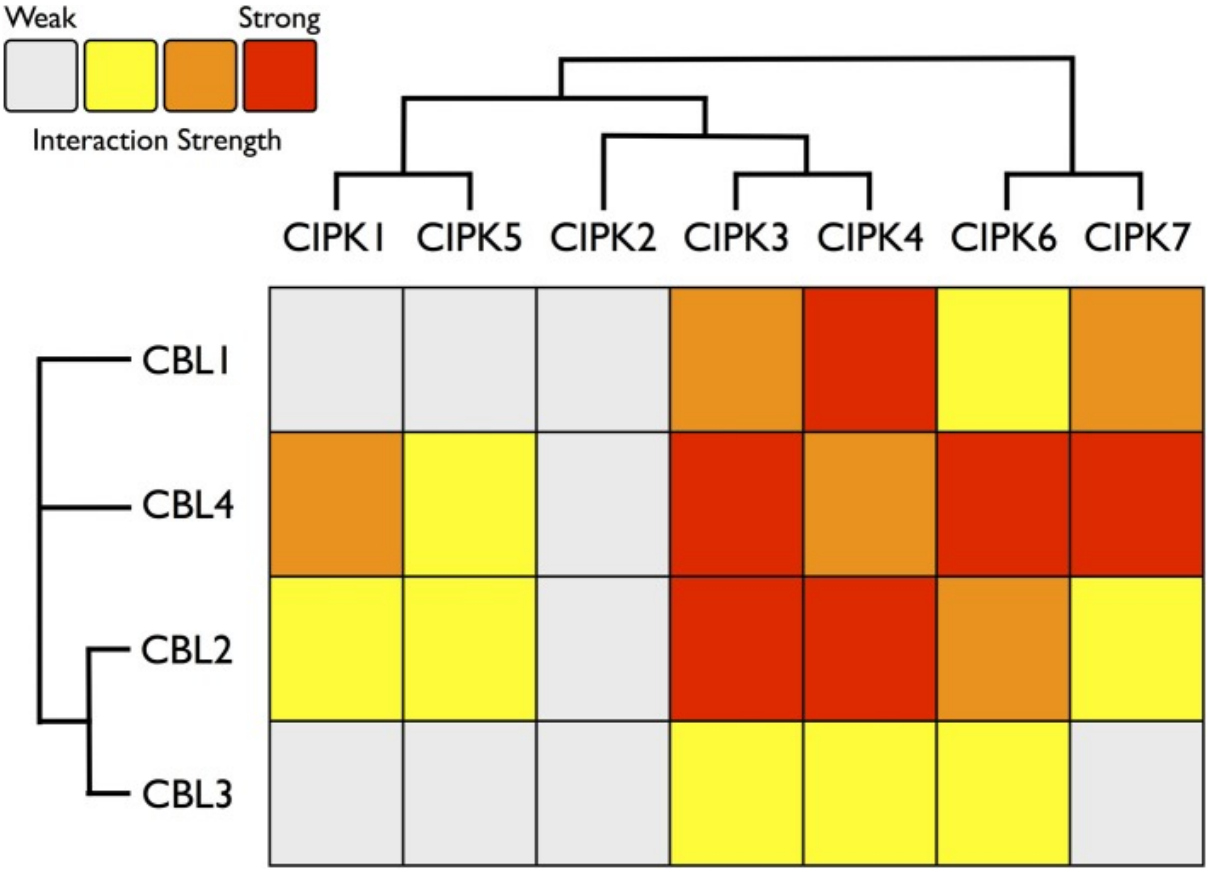


Figure 3.8: Heat map summarizing yeast two-hybrid (Y2H) results for all *Physcomitrella* CBL and CIPK combinations. Overall results of Y2H growth assays are presented as a heat map. Red boxes indicate vigorous growth on -LTHA plates; orange boxes indicate weaker growth on -LTHA plates. Yellow boxes indicate robust growth on -LTH plates but no growth on -LTHA plates. Gray boxes indicate weak growth on -LTH plates, but each CBL-CIPK interaction conferred better growth than the empty vector (EV) control. Inferred phylogenetic relationships of *Physcomitrella* CBLs and CIPKs are indicated by cladogram and described in the main text.



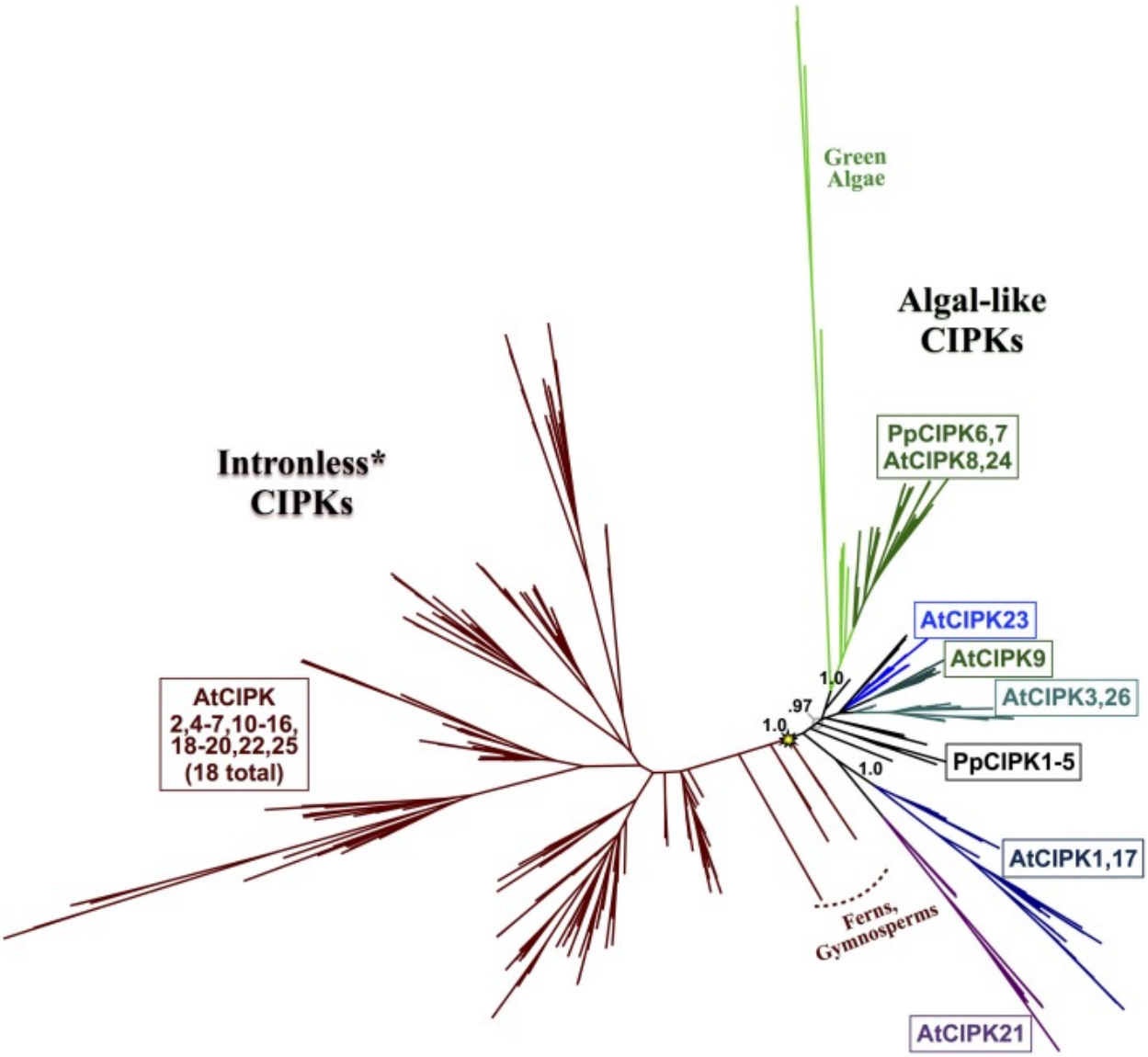


Figure 3.9: ML phylogenetic tree derived from protein MSA of all CIPKs identified in this study. Confidence scores (aLRT) are shown for select clades. CIPKs from green algae phylogenetically cluster with land plant CIPKs, including *Physcomitrella* CIPK6 and CIPK7. Remaining *Physcomitrella* CIPKs cluster with Arabidopsis CIPK1, 17, and 21, which contain multiple introns, and a clade of "intronless" *CIPKs* (although *AtCIPK16* has gained one intron) derived from an inferred reverse transcription event (yellow star). (*Although AtCIPK16 has gained one intron.)

Figure 3.10: Close-up of the "green algal-type" CIPK clade. Confidence scores (aLRT) are shown for each clade. Phylogenetic evidence strongly supports the existence of a clade (aLRT = 1.0) containing all CIPK homologs identified from chlorophyte and charophyte green algae, as well as two CIPKs each from *Physcomitrella* (yellow dots) and Arabidopsis (green dots). *Physcomitrella* CIPK6 and CIPK7 are recent paralogs and sister to one another in our analyses. In contrast, Arabidopsis CIPK8 and CIPK24 each have clear orthologs in other sequenced angiosperms, and these clades appear to have arisen from a gene duplication that occurred around the time of divergence of angiosperms (arrow).

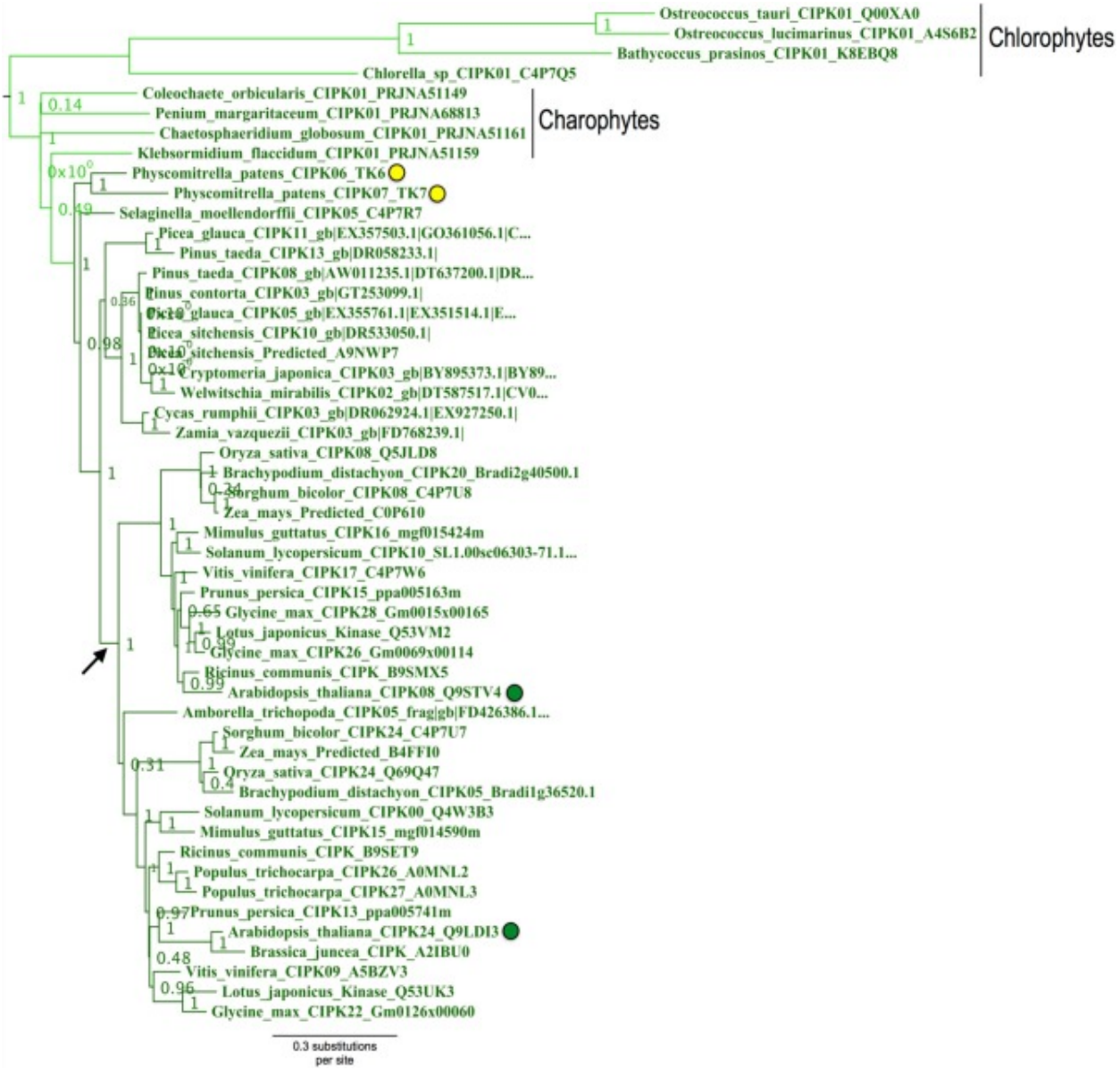
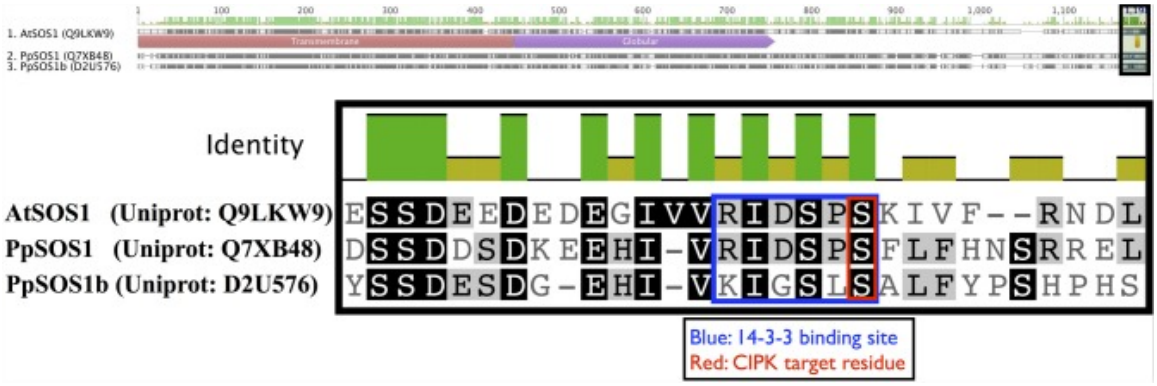


Figure 3.11: Key regulatory residues at the C-terminus of Arabidopsis SOS1 (AtSOS1) are conserved in *Physcomitrella* homologs. The full-length protein sequence alignment is shown on top with the zoomed in region indicated by a shaded box. Arabidopsis CIPK24 (SOS2) phosphorylates the serine (S) residue marked in red in AtSOS1 and facilitates binding by a 14-3-3 protein. In Arabidopsis, these mechanisms are critical to plant sodium tolerance. Strong sequence conservation suggest similar mechanisms may be in place in *Physcomitrella*, though the cognate CBL-CIPK pair is currently unknown.



Based on our results, we posit that *Physcomitrella* CIPK6 and CIPK7 and Arabidopsis CIPK8 and CIPK24 (SOS2) most closely resemble the ancestral or "green algal-type" CIPK and, due to their orthology, most likely to reflect ancestral function(s) of the CBL-CIPK network.

Arabidopsis CIPK8 is believed to be a positive regulator of the low-affinity phase of the primary nitrate response and has been implicated in glucose sensing, although mechanistic details are unknown at this time (Hu *et al.* 2009). Arabidopsis CIPK24, the first functionally characterized CIPK, plays a critical function in sodium tolerance through CBL4(SOS3)-modulated phosphorylation of the Na^+/H^+ exchanger SOS1. There is substantial evidence that orthologs of CBL4 and CIPK24 in other flowering plant lineages have similar functions (Martdnez-Atienza *et al.* 2007; Tang *et al.* 2010). Given the phylogenetic proximity of Arabidopsis CIPK24 to green algal CIPKs, future work will test whether green algal CIPKs, and *Physcomitrella* CIPK6 and CIPK7, function in Na^+/K^+ homeostasis or possibly more broadly regulate ion transport. It has already been established that two orthologs of SOS1 in *Physcomitrella* (PpSOS1 and PpSOS1b) are required for proper K^+/Na^+ ratios and sodium tolerance (Quintero *et al.* 2011). Interestingly, a 6 AA C-terminal motif of AtSOS1 that is a phosphorylation substrate of CIPK24 and a 14-3-3 protein-binding site is 100% identical to PpSOS1 and 50% identical to PpSOS1b, and the target serine is conserved in both homologs (Figure 3.11). *Physcomitrella* SOS1 has further been shown to confer enhanced NaCl tolerance when heterologously expressed in yeast, and the effect is strengthened by coexpression with Arabidopsis CBL4 and CIPK24 (Fraile-Escanciano *et al.* 2010). Collectively, these observations suggest that the SOS pathway is conserved across land plants and may be conserved among some green algal lineages. Functional molecular analyses of

CBLs and CIPKs in early-diverging plant and algal lineages could provide core insights and clarify the increasingly complex picture of calcium-regulated abiotic stress responses in Arabidopsis and agricultural species.

Conclusions.

Prior publications (e.g. Weinl *et al.* 2009; Batistič and Kudla, 2009) have mentioned the apparent expansion of the CBL-CIPK network in terms of the total numbers of CBLs and CIPKs found in algae and early diverging plants compared to their angiosperm counterparts. Here, we present phylogenetic evidence that the CBL-CIPK network has expanded independently in multiple plant lineages, including mosses and angiosperms. It appears that the common ancestor of mosses and vascular plants likely contained three CBLs distinguishable by N-terminal localization motifs, which likely are synapomorphies among ancient CBL subfamilies. We have identified a clade of CIPKs containing all green algal homologs and two representatives from *Physcomitrella* and Arabidopsis. Phylogenetic analysis demonstrates that the *Physcomitrella* and Arabidopsis members of this clade are the products of independent gene duplications and the earliest land plants likely contained a single homolog from this clade. The concurrent pairing of *CBLs* and *CIPKs* in available genomes and transcriptomes, the striking conservation of the NAF domain, and our Y2H results all point towards a physically and functionally connected CBL-CIPK network across plants and algae.

The function(s) of CBL-CIPK pairs found in green algae remains an open and intriguing question, and our identification of charophyte CBL-CIPK pairs expands the list of potential models for this inquiry. The conspicuous expansion of the network in several land plant lineages appears to have been driven largely by WGDs, and we hypothesize that duplicated members were adapted for novel signaling pathways and precise roles in particular cells and tissues. Research on molecular processes modulated by CBLs and CIPKs has intensified in recent years, and researchers are beginning to investigate CBL-CIPK functions in non-model angiosperm species. The field is prime for investigation of CBL-CIPK functions in earlier diverging land plants, and research in this area will enhance our understanding of the molecular evolutionary basis of the colonization of land by plants.

Sources Cited:

Albrecht V., Ritz O., Linder S., Harter K., & Kudla J. (2001). The NAF domain defines a novel protein–protein interaction module conserved in Ca^{2+}-regulated kinases. *The EMBO Journal 20*, 1051-1063.

Anisimova M., & Gascuel O. (2006). Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Systematic Biology* 55, 539-552.

Batistic O., Sorek N., Schü O. S., Yalovsky S., & Kudla J. (2008). Dual fatty acyl modification determines the localization and plasma membrane targeting of CBL/CIPK Ca^{2+} signaling complexes in Arabidopsis. *The Plant Cell*, *20*(5), 1346-1362.

Batistič, O., and Kudla, J. (2009). Plant calcineurin B-like proteins and their interacting protein kinases. *Biochimica et Biophysica Acta* 1793, 985–992.

Batistic O., Waadt R., Steinhorst L., Held K., & Kudla J. (2010). CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. *The Plant Journal* 61, 211–222.

Batistic O., Kim K.N., Kleist T., Kudla J., & Luan S. (2011). The CBL-CIPK Network for decoding calcium signals in plants. In S. Luan (Ed.), *Coding and Decoding of Calcium Signals in Plants* (pp. 235-258). Springer.

Batistič O. (2012). Genomics and localization of the Arabidopsis DHHC-cysteine-rich domain S-acyltransferase protein family. *Plant Physiology* 160, 1597–1612.

Batistič O., Rehers M., Akerman A., Schlücking K., Steinhorst L., Yalovsky S., & Kudla J. (2012). S-acylation-dependent association of the calcium sensor CBL2 with the vacuolar membrane is essential for proper abscisic acid responses. *Cell research* 22, 1155-1168.

Bhatnagar R. S., & Gordon J. I. (1997). Understanding covalent modifications of proteins by lipids: where cell biology and biophysics mingle. *Trends in Cell Biology 7*, 14-20.

Bijlmakers M.-J., & Marsh M. (2003). The on–off story of protein palmitoylation. *Trends in cell biology*, *13* 32-42.

Bowman J. L., Floyd S. K., & Sakakibara K. (2007). Green genes—comparative genomics of the green branch of life. *Cell* 129, 229–234.

Chang S., Puryear J., & Cairney J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter*, *11*(2), 113-116.

Cheong Y. H., Pandey G. K., Grant J. J., Batistic O., Li L., Kim B. G., ... & Luan S. (2007). Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. *The Plant Journal* 52, 223-239.

Clapham D. E. (2007). Calcium signaling. *Cell 131*, 1047-1058.

Cui L., Wall P. K., Leebens-Mack J. H., Lindsay B. G., Soltis D. E., Doyle J. J., ... & Ma H. (2006). Widespread genome duplications throughout the history of flowering plants. *Genome Research* 16, 738-749.

Cyert M. S. (2001). Genetic analysis of calmodulin and its targets in *Saccharomyces cerevisiae*. *Annual Review of Genetics*, *35*, 647-672.

D'Angelo C., Weinl S., Batistic O., Pandey G. K., Cheong Y. H., Schültke S., ... & Kudla J. (2006). Alternative complex formation of the Ca2+-regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in Arabidopsis. *The Plant Journal* 48, 857-872.

Dunphy J. T., & Linder M. E. (1998). Signalling functions of protein palmitoylation. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1436, 245-261.

Eisen J. A., & Wu M. (2002). Phylogenetic analysis and gene functional predictions: phylogenomics in action. *Theoretical Population Biology* 61, 481-487.

Ellegren H. (2004). Microsatellites: simple sequences with complex evolution. *Nature Reviews Genetics* 5, 435-45.

Engelman D. M. (2005). Membranes are more mosaic than fluid. *Nature* 438, 578-580.

Evans N. H., McAinsh M. R., & Hetherington A. M. (2001). Calcium oscillations in higher plants. *Current Opinion in Plant Biology* 4, 415-420.

Filichkin S. A., Priest H. D., Givan S. A., Shen R., Bryant D. W., Fox S. E., ... & Mockler T. C. (2010). Genome-wide mapping of alternative splicing in *Arabidopsis thaliana*. *Genome Research* 20, 45-58.

Fogelson A. L., & Zucker R. S. (1985). Presynaptic calcium diffusion from various arrays of single channels: implications for transmitter release and synaptic facilitation. *Biophysical Journal* 48, 1003-1017.

Fraile-Escanciano A., Kamisugi Y., Cuming A. C., Rodríscan-Navarro A., & Benito

B. (2010). The SOS1 transporter of *Physcomitrella patens* mediates sodium efflux *in planta*. *New Phytologist* 188, 750-761.

Gilroy S., Bethke P. C., & Jones R. L. (1993). Calcium homeostasis in plants. *Journal of Cell Science* 106, 453-461.

Graham L. E. (1996). Green algae to land plants: an evolutionary transition. *Journal of Plant Research* 109, 241-251.

Guindon S., Delsuc F., Dufayard J.F., & Gascuel O. (2009). Estimating maximum likelihood phylogenies with PhyML. In *Bioinformatics for DNA Sequence Analysis* (pp. 113-137). Springer.

Guo Y., Halfter U., Ishitani M., & Zhu J. K. (2001). Molecular characterization of functional domains in the protein kinase SOS2 that is required for plant salt tolerance. *The Plant Cell* 13, 1383-1400.

Hemsley P. A., & Grierson C. S. (2008). Multiple roles for protein palmitoylation in plants. *Trends in Plant Science* 13, 295-302.

Hirotsune S., Yoshida N., Chen A., Garrett L., Sugiyama F., Takahashi S., ... & Yoshiki A. (2003). An expressed pseudogene regulates the messenger-RNA stability of its homologous coding gene. *Nature* 423, 91-96.

Ho C.H., Lin S.H., Hu H.C., & Tsay Y.F. (2009). CHL1 functions as a nitrate sensor in plants. *Cell* 138, 1184-1194.

Hrabak E. M., Chan C. W., Gribskov M., Harper J. F., Choi J. H., Halford N., ... & Harmon A. C. (2003). The Arabidopsis CDPK-SnRK superfamily of protein kinases. *Plant Physiology* 132, 666-680.

Hu H., Wang Y., & Tsay Y. (2009). AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. *The Plant Journal* 57, 264-278.

Jain E., Bairoch A., Duvaud S., Phan I., Redaschi N., Suzek B. E., ... & Gasteiger E. (2009). (2009). Infrastructure for the life sciences: design and implementation of the UniProt website. *BMC Bioinformatics* 10, 136.

Jeffares D. C., Mourier T., & Penny D. (2006). The biology of intron gain and loss. *Trends in Genetics* 22, 16-22.

Jiao Y., Wickett N. J., Ayyampalayam S., Chanderbali A. S., Landherr L., Ralph P. E., ... & Leebens-Mack J. (2011). Ancestral polyploidy in seed plants and angiosperms. *Nature* 473, 97-100.

Kenrick P., & Crane P. R. (1997). The origin and early evolution of plants on land. *Nature* 389, 33-39.

Kim B. G., Waadt R., Cheong Y. H., Pandey G. K., Dominguez-Solis J. R., Schültke S., ... & Luan S. (2007). The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabidopsis. *The Plant Journal* 52, 473-484.

Kolukisaoglu Ü., Weinl S., Blazevic D., Batistic O., & Kudla J. (2004). Calcium sensors and their interacting protein kinases: genomics of the Arabidopsis and rice CBL-CIPK signaling networks. *Plant Physiol*ogy 134, 43–58.

Korneev S. A., Park J. H., & O'Shea M. (1999). Neuronal expression of neural nitric oxide synthase (nNOS) protein is suppressed by an antisense RNA transcribed from an NOS pseudogene. *Journal of Neuroscience* 19, 7711-7720.

Kudla J., Xu Q., Harter K., Gruissem W., & Luan S. (1999). Genes for calcineurin B-like proteins in Arabidopsis are differentially regulated by stress signals. *Proceedings of the National Academy of Sciences U.S.A.* 96, 4718-4723.

Levental I., Grzybek M., & Simons K. (2010). Greasing their way: lipid modifications determine protein association with membrane rafts. *Biochemistry* 49, 6305-6316.

Lewis L. A., & McCourt R. M. (2004). Green algae and the origin of land plants. *American Journal of Botany* 91, 1535-1556.

Li C., Pan W., Braunewell K. H., & Ames J. B. (2011). Structural analysis of Mg^{2+} and Ca^{2+} binding, myristoylation, and dimerization of the neuronal calcium sensor and visinin-like protein 1 (VILIP-1). *Journal of Biological Chemistry* 286, 6354-6366.

Li L., Kim B. G., Cheong Y. H., Pandey G. K., & Luan S. (2006). A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in Arabidopsis. *Proceedings of the National Academy of Sciences U.S.A.* 103, 12625-12630.

Liu J., Ishitani M., Halfter U., Kim C.-S., & Zhu J.-K. (2000). The *Arabidopsis thaliana SOS2* gene encodes a protein kinase that is required for salt tolerance. *Proceedings of the National Academy of Sciences U.S.A.* 97, 3730-3734.

Liu J., & Zhu J. K. (1998). A calcium sensor homolog required for plant salt tolerance. *Science* 280, 1943-1945.

Luan S. (2009). The CBL–CIPK network in plant calcium signaling. *Trends in Plant Science* 14, 37-42.

Luan S., Kudla J., Rodriguez-Concepcion M., Yalovsky S., & Gruissem W. (2002). Calmodulins and calcineurin B–like proteins: calcium sensors for specific signal response coupling in plants. *The Plant Cell* 14, S389–S400.

Martínez-Atienza J., Jiang X., Garciadeblas B., Mendoza I., Zhu J. K., Pardo J. M., & Quintero F. J. (2007). Conservation of the salt overly sensitive pathway in rice. *Plant Physiology* 143, 1001-1012.

McCormack E., & Braam J. (2003). Calmodulins and related potential calcium sensors of Arabidopsis. *New Phytologist* 159, 585-598.

McCormack E., Tsai Y. C., & Braam J. (2005). Handling calcium signaling: Arabidopsis CaMs and CMLs. *Trends in Plant Science* 10, 383-389.

Mumby S. M. (1997). Reversible palmitoylation of signaling proteins. *Current Opinion in Cell Biology* 9, 148-154.

Nagae M., Nozawa A., Koizumi,N., Sano H., Hashimoto H., Sato M., & Shimizu T. (2003). The crystal structure of the novel calcium-binding protein AtCBL2 from *Arabidopsis thaliana*. *Journal of Biological Chemistry* 278, 42240-42246.

Pandey G. K., Cheong Y. H., Kim B. G., Grant J. J., Li L., & Luan S. (2007). CIPK9: a calcium sensor-interacting protein kinase required for low-potassium tolerance in Arabidopsis. *Cell Research* 17, 411-421.

Pandey G. K., Grant J. J., Cheong Y. H., Kim B. G., Li L., & Luan S. (2008). Calcineurin-B-like protein CBL9 interacts with target kinase CIPK3 in the regulation of ABA response in seed germination. *Molecular Plant* 1, 238-248.

Pittermann J. (2010). The evolution of water transport in plants: an integrated approach. *Geobiology* 8, 112-139.

Quan R., Lin H., Mendoza I., Zhang Y., Cao W., Yang Y., ... & Guo Y. (2007). SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress. *The Plant Cell* 19, 1415-1431.

Quintero F. J., Martinez-Atienza J., Villalta I., Jiang X., Kim W. Y., Ali Z., ... & Pardo J. M. (2011). Activation of the plasma membrane Na/H antiporter Salt-Overly-Sensitive 1 (SOS1) by phosphorylation of an auto-inhibitory C-terminal domain. *Proceedings of the National Academy of Sciences U.S.A.* 108, 2611-2616.

Ren X. L., Qi G. N., Feng H. Q., Zhao S., Zhao S. S., Wang Y., & Wu W. H. (2013). Calcineurin B-like protein CBL10 directly interacts with AKT1 and modulates K^+ homeostasis in Arabidopsis. *The Plant Journal* 74, 258-266.

Rensing S. A., Lang D., Zimmer A. D., Terry A., Salamov A., Shapiro H., ... & Rushton P. J. (2008). The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* 319, 64-69.

Rensing S. A., Beike A. K., & Lang D. (2013). Evolutionary importance of generative polyploidy for genome evolution of haploid-dominant land plants. In *Plant Genome Diversity Volume 2* (pp. 295-305). Springer.

Rensing S. A., Ick J., Fawcett J. A., Lang D., Zimmer A., Van de Peer Y., & Reski R. (2007). An ancient genome duplication contributed to the abundance of metabolic genes in the moss *Physcomitrella patens*. *BMC Evolutionary Biology* 7, 130.

Roberts W. M. (1994). Localization of calcium signals by a mobile calcium buffer in frog saccular hair cells. *The Journal of Neuroscience* 14, 3246-3262.

Shi H., Ishitani M., Kim C., & Zhu J.-K. (2000). The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na^+/H^+ antiporter. *Proceedings of the National Academy of Sciences U.S.A* 97, 6896-6901.

Shi J., Kim K. N., Ritz O., Albrecht V., Gupta R., Harter K., ... & Kudla J. (1999). Novel protein kinases associated with calcineurin B–like calcium sensors in Arabidopsis. *The Plant Cell* 11, 2393-2405.

Stanke M., Steinkamp R., Waack S., & Morgenstern B. (2004). AUGUSTUS: a web server for gene finding in eukaryotes. *Nucleic Acids Research* 32, W309-W312.

Tam O. H., Aravin A. A., Stein P., Girard A., Murchison E. P., Cheloufi S., ... & Hannon G. J. (2008). Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* 453, 534-538.

Tang R. J., Liu H., Bao Y., Lv Q. D., Yang L., & Zhang H.X. (2010). The woody plant poplar has a functionally conserved salt overly sensitive pathway in response to salinity stress. *Plant Molecular Biology* 74, 367-380.

Tang R. J., Liu H., Yang Y., Yang L., Gao X. S., Garcia V. J., ... & Zhang H. X. (2012). Tonoplast calcium sensors CBL2 and CBL3 control plant growth and ion homeostasis through regulating V-ATPase activity in Arabidopsis. *Cell Research* 22, 1650-65.

Tang R. J., Yang Y., Yang L., Liu H., Wang C. T., Yu M. M., ... & Zhang H. X. (2013). Poplar calcineurin B-like proteins PtCBL10A and PtCBL10B regulate shoot salt tolerance through interaction with PtSOS2 in the vacuolar membrane. *Plant, Cell and Environment* 37, 573-588.

Timme R., & Delwiche C. (2010). Uncovering the evolutionary origin of plant molecular processes: comparison of *Coleochaete* (Coleochaetales) and *Spirogyra* (Zygnematales) transcriptomes. *BMC Plant Biology* 10, 96.

Timme R. E., Bachvaroff T. R., & Delwiche C. F. (2012). Broad phylogenomic sampling and the sister lineage of land plants. *PLoS One* 7, e29696.

Verret F., Wheeler G., Taylor A. R., Farnham G., & Brownlee C. (2010). Calcium channels in photosynthetic eukaryotes: implications for evolution of calcium-based signalling. *New Phytologist* 187, 23-43.

Weinl S., & Kudla J. (2009). The CBL-CIPK Ca^{2+}-decoding signaling network: function and perspectives. *New Phytologist* 184, 517-528.

Wheeler G. L., & Brownlee C. (2008). Ca^{2+} signalling in plants and green algae–changing channels. *Trends in Plant Science*, 13, 506-514.

Witman G. B. (1993). *Chlamydomonas* phototaxis. *Trends in Cell Biology* 3, 403-408.

Xu, J., Li, H. D., Chen, L. Q., Wang, Y., Liu, L. L., He, L. *et al.* (2006). A protein kinase, interacting with two calcineurin b-like proteins, regulates K^+ transporter AKT1 in Arabidopsis. *Cell* 125, 1347-1360.

Zimmer A. D., Lang D., Buchta K., Rombauts S., Nishiyama T., Hasebe M., ... & Reski R. (2013). Reannotation and extended community resources for the genome of the non-seed plant *Physcomitrella patens* provide insights into the evolution of plant gene structures and functions. *BMC Genomics* 14, 498.

Chapter 4: Advancement of genetic transformation techniques to investigate calcium signaling processes in the moss *Physcomitrella patens*.

Preface.

The publication of the *Physcomitrella* genome sequence, referenced in Chapter 1, has enabled genomic studies of bryophytes for the first time. In order to serve as a useful model for functional genomics, a biological system must be amenable to genetic manipulation. *Physcomitrella* emerged as the major bryophyte model system owing to the unexpected observation that it can undergo gene targeting at rates drastically higher than observed with other plants. Nonetheless, discussions at scientific conferences and would suggest that many researchers have struggled to establish procedures for genetic transformation of *Physcomitrella* in their laboratories, with the aim to facilitate molecular genetic stuides in moss. The following chapter provides a set of guidelines for culturing and transforming *Physcomitrella patens*. It is arranged into four sections. Section 1 providues an introduction to mosses as model systems for plant biology. Section 2 describes procedures for obtaining targeted transformants in *Physcomitrella* via protoplast transformation and mentions pitfalls in currently published protocols. Section 3 describes genetic transformation of *Physcomitrella* via biolistics, which is not suitable for gene targeting but is desirable for non-targeted delivery of transgenes and significantly accelerates the generation of stable transformants. Section 4 contains descriptions of all growth media used for *Physcomitrella* culture and genetic transformation.

Section 1: Introduction.

Background: Mosses as Experimental Systems.

Cladistic treatments variously recognize one of the three living types of bryophyte, mosses, liverworts or hornworts, as nearest sister to the clade of all the other land plants. For that reason, mosses hold great bearing on our understanding of all aspects of land plant biology evolution. Now recognized as an early-diverging lineage of plants, their earlier status as a lower grade of plant life means that mosses have a rich history of use in laboratory research. In the 1920s, Fritz von Wettstein used various mosses, including *Funaria hygrometrica*, to show that these plants too obeyed the laws of Mendelism (reviewed by von Wettstein, 1932). *Funaria* was later adopted by the eminent bryologist Martin Bopp and served as the major experimental platform for developing much of our current understanding of the fundamental aspects of moss physiology and development (reviewed by Bopp, 2000).

The demonstration that protoplasts of the closely related moss *Physcomitrella* could go undergo gene targeting via homologous recombination (Schaefer & Zrÿd, 1997) in a manner akin to yeast has drawn a great deal of interest from the plant biology community. In 2008, *Physcomitrella patens* became the first moss to have a published genome sequence (Rensing *et al*. 2008). Since, it has become a major platform for comparative and functional genomics and been adopted by a growing number of labs for molecular genetic work.

With recent advances in genome sequencing, projects are underway to sequence and annotate the genomes of *Funaria hygrometrica* (Szovenyi *et al*. 2013), the dioiceous (i.e. bisexual, with different colonies giving producing to either antheridia or archegonia) species *Ceratodon purpureus* (Szovenyi *et al*. 2015), and multiple species in the ecologically important genus *Sphagnum* (peat moss) (Weston *et al*. 2014). The genus *Syntrichia* (syn. *Tortula*) has been intensively studied in an ecological context, and transcriptome-level studies are also being pursued for members of this genus (Li *et al*. 2015). In order to encourage and facilitate molecular genetic studies in *Physcomitrella* and other mosses, this work provides a set of protocols that detail different strategies for moss genetic transformation, along with guidelines to assist researchers who are new to working with *Physcomitrella* or who are pursuing transformation in other moss lineages.

Transformation of *Physcomitrella patens*.

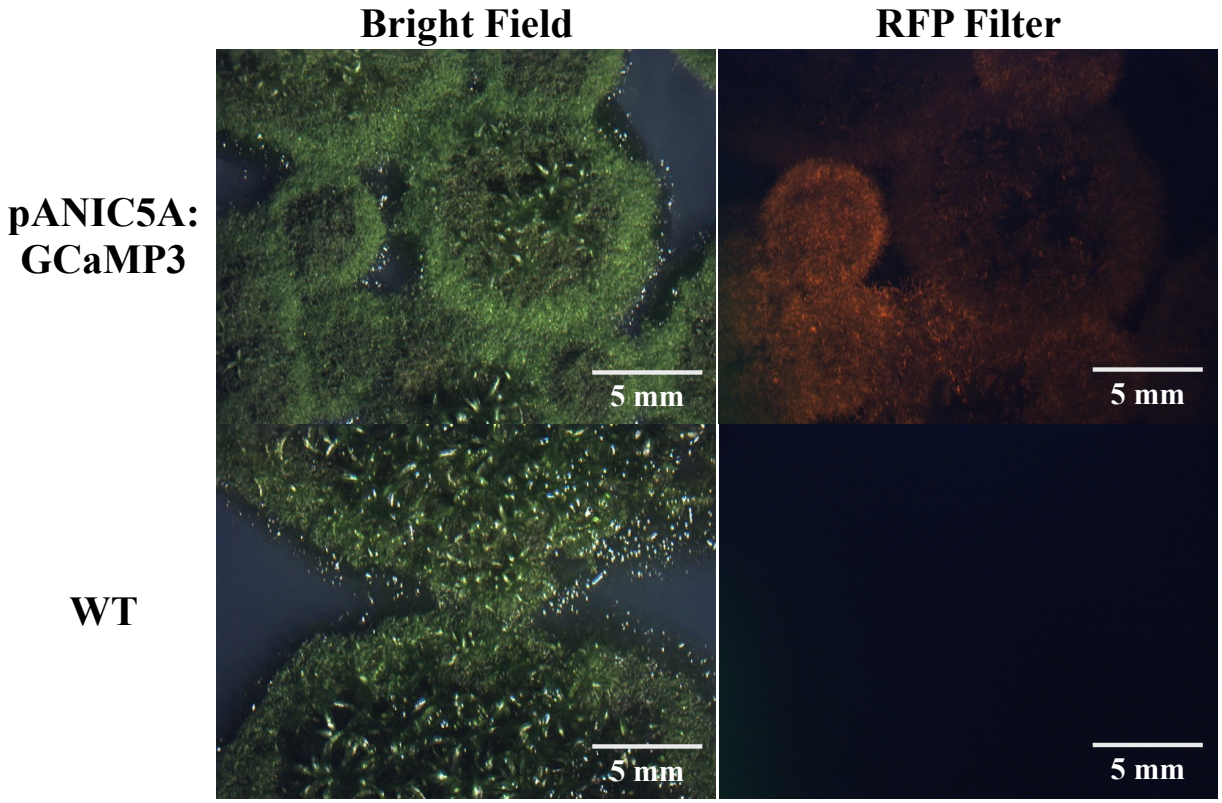
Aside from its capacity for gene targeting, *Physcomitrella* shows another unusual behavior during transformation that differs from patterns observed in flowering plants. In flowering plants, genetically transformed cells fall into two modalities: stable transformants that integrate into plant chromosomes and transient transformants that show transgene expression for a short duration – usually a few days – and promptly lose the transgene construct. In *Physcomitrella*, a third modality is commonly observed: "semi-stably" transformed plants that apparently replicate transgene constructs

extrachromosomally at sufficient rates to spread mitotically (Ashton *et al*. 2000). Such semi-stable transformants can be obtained from protoplast or biolistic transformation and can be maintained for weeks without selection or years with continuous selection. This unique feature presents a quandary for analyzing chromosomal integrants – do stably transformed cells integrate into chromosomes promptly following transformation, or does integration occur following a period of extrachromosomal replication? This unusual ability to maintain extrachromosomal DNA also introduces additional complexities for analyzing putative gene targeting events – how might one accurately estimate the rate of targeting without knowing whether integration occurs promptly or following a period of extrachromosomal replication, and what is the fate of replaced genomic regions once excised from the chromosome? Moreover, could the phenomena of extrachromosomal replication and gene targeting, both unique among studied land plants, be mechanistically linked? Chromosomal integration usually occurs either by non-homologous end-joining (NHEJ) leading to random integration or by homologous recombination leading to targeted integration; the apparent rate of gene targeting for a particular organism, cell type, and transformation method is a function of these two rates.

An interesting feature of *Physcomitrella* protoplast transformation protocols is that transformed cells are placed on antibiotic selection, removed from selection for a period of usually one to two weeks, then subjected to a second round of selection. This two-stage selection strategy may reflect delayed integration of constructs following extrachromosomal replication or may simply facilitate the identification of semi-stable transformants. An alternative strategy to delineate stably versus semi-stably transformed cells is to include a visual marker such as a fluorescent protein that can be non-invasively monitored. We have adopted red fluorescent protein (RFP) for this purpose, which can be easily monitored in *Physcomitrella* protonema using a Zeiss stereo Lumar v12 (Carl Zeiss) or similar microscope (Figure 4.1).

Protocols for transformation of *Physcomitrella* protoplasts using polyethylene glycol (PEG) and heat shock (e.g. Cove *et al*., 2009b) have been widely adopted for gene targeting; however, we are unaware of published data to clarify whether gene targeting is a unique outcome of protoplast transformation or if it can occur at reasonably high rates with other transformation methods such as particle bombardment. Transformation of *Phycomitrella* using *Agrobacterium* has been reported but is typically achieved using protoplasts (e.g. Cove *et al*., 2009c) rather than intact cells, although the latter has also been reported (Li *et al*., 2010). Interestingly, Agrobacterium-mediated transformation reportedly leads to random integration of T-DNA molecules (Cove *et al*., 2009c), in contrast to other methods. This suggests that *Agrobacterium*-mediated transformation in some way alters the relative rates of HR and NHEJ. We are unaware of any data to clarify whether *Agrobacterium*-mediated transformation of *Physcomitrella* yields semi-stable transformants like other transformation methods. Recent data from Arabidopsis suggest that T-DNA integration may follow a pathway that is distinct from NHEJ or HR and mediated by distinct genetic components (Park *et al*. 2015), providing one plausible explanation for the random integration of T-DNAs in *Physcomitrella*.

Figure 4.1: Use of red fluorescent protein (RFP) for non-invasive monitoring of transgene status in the moss *Physcomitrella patens*. Vector containing an RFP expression constuct was delivered by biolistics. Images were taken using a Zeiss stereo lumar microscope. RFP expression is driven by the rice actin 1 (OsAct1) promoter and shows strong expression in protonema.



Based on observations made during this doctoral dissertation research, it appears that biolistic transformation is not suitable for gene targeting or replacement. During the course of molecular genetic investigation, we implemented a four-piece multisite gateway system to assemble knockout constructs containing terminal approximately 1 kilobase (Kb) flanks cloned from genomic DNA (gDNA) with two intervening marker constructs positioned between the flanks. The marker gene constructs contain an antibiotic resistance gene (*NPTII* or *HPH*) visual selection mark er (RFP or green fluorescent protein, GFP). The constructs were linearized by restriction digest, purified with chloroform, ethanol precipitated, and resuspended in TE buffer. Among 50 stably transformed lines that showed red fluorescent protein (RFP) expression using a Zeiss Stereo Lumar v12 (Carl Zeiss), none were found to contain an insertion at or replacement of the targeted loci (data not shown). Other groups have noted that elimination of the vector backbone by gel extraction of the restriction digest product or, preferably, by PCR amplification of knockout constructs improves rates of gene targeting (personal communication with Steven Theg, UC-Davis), though we are unaware of quantitative data to support this assertion.

Although *Physcomitrella* has been compared to budding yeast (*Saccharomyces cerevisiae*) because of its capacity to undergo gene targeting, the guidelines to achieve gene targeting are quite different in the two organisms. In budding yeast, oligonucleotide primers with 5' extensions in the range of 25 – 35 nucleotides that are identical to targeted sites in the yeast genome are used for PCR-amplification of marker genes, allowing for simple generation of knockout constructs. In *Physcomitrella*, homologous flanks shorter than approximately 500 nucleotides reportedly lead to inefficient gene targeting, and 1 KB flanks have been recommended for efficient gene targeting (Sawahel *et al*. 1992; Cho *et al*. 1999; Cove *et al*. 2009a; Šmídková *et al*. 2010). Furthermore, budding yeast can be easily transformed without digestion of the cell wall, whereas cell wall digestion during *Physcomitrella* transformation protocols introduces the potential for protoplast fusion and aberrancies in ploidy. Although roughly 50% gene targeting efficiency among stable transformants – similar to rates achieved in budding yeast – has been reported in *Physcomitrella*, lower rates are often achieved in practice. Moreover, unlike in budding yeast, apparent cases of gene targeting are often accompanied by concatemers and rearrangements. A recent systematic analysis of stable *Physcomitrella* transformants provided evidence for frequent aberrancies in gene targeting and suggested that ectopic targeting, or 'hit-and-run' integration, is a common occurence (Wendeler *et al*. 2015).

Whereas gene targeting is an attractive feature of *Physcomitrella* as a model plant, non-targeted delivery of genetically encoded probes, modified genes or heterologous genes is also commonly required for molecular genetic investigation. Protoplast transformation by PEG + heat shock is generally held to be inefficient for delivery of non-targeted DNA constructs and rarely leads to stable transgene expression. The construction of gene targeting constructs and selection of a native locus for targeting, as well as challenges that can arise working with protoplasts, may act as deterrent for these efforts. Transformation of *Physcomitrella* protoplasts via biolistic transformation provides an alternative method for transgene delivery. For reasons that are currently unclear, promoters used for transgene expression in grasses tend to also be effective for driving transgene expression in *Physcomitrella*. Like grasses, the popular cauliflower mosaic virus 35S promoter does not reliably lead to strong or stable expression (Horstmann *et al*. 2004). We have adopted biolistic vectors designed for use in switchgrass (*Panicum virgatum*) and other monocots (Mann *et al*., 2012) that have proven to be effective for transgene expression in *Physcomitrella*. In order to obtain transgenic lines containing genetically encoded calcium indicators (GECIs) and to pursue gain-of-function analysis of putative moss signaling proteins, we generated a large number of stably transformed *Physcomitrella* lines and developed an accelerated and highly effective protocol for the biolistic transformation of protonemal cells or spores.

Prospects for Stable Transformation of Other Mosses.

Many characteristics of moss biology make them attractive laboratory models. Their small size enables cultivation in sterile petri dishes, and large populations can be maintained in a single growth chamber. Their tissues are generally only a single cell-layer thick, making them ideally suited for microscopy. In most cases, mosses can be readily subcultured by transfer of vegetative tissue. Many mosses produce vegetative propagules, some of which are desiccation tolerant and could be useful for storage of moss lines. Mosses typically produce large number of spores (e.g. thousands to hundreds of thousands per capsule), which could be leveraged for genetic analysis. Despite its demonstrated utility for molecular biology, *Physcomitrella* has certain undesirable characteristics for a model moss. It is monoicous, which greatly complicates genetic crosses; and the sporophytes are borne on exceptionally short seta, which retards the collection of large number of spore capsules. Moreover, despite being widely touted for its haploid-dominant life cycle, the *Physcomitrella* genome is heavily influenced by a polyploidization event (Rensing *et al*. 2008), and many genes have highly similar paralogs that show functional redundancy.

Axenic cultures of mosses are generally easy to establish because unopened spore capsules can be surface sterilized prior to releasing the spores. Moreover, moss spores are single-celled and are ideally suited as targets for genetic transformation. Transformation of fern spores to generate stable transgenics has been recently reported (Muthukumar *et al*. 2013). Mosses produce a wide assortment of asexual propagules that may also be suitable transformation targets. In particular, protonemal propagules known as brachycytes or brood cells, which are stimulated by application of the phytohormone abscisic acid (ABA), may be suitable transformation targets in various moss species. Looking forward, it is our carefully considered opinion that the field of plant biology would benefit from the establishment of additional moss lineages as model organisms for laboratory work.

Section 2: Protoplast transformation procedure for gene targeting.

Overview.

Physcomitrella protoplasts can be readily obtained from young protonemal cells (i.e. chloronema) by enzymatic digestion of the cell walls using a commerically available enzyme cocktail marketed as Driselase (Sigma). Protoplasts can be quantified using a haemocytometer and adjusted to a standard density. Protoplasts can be stimulated to take up co-incubated DNA by a combination of heat shock, administered using a water bath or hot plate, and osmotic shock, administered using PEG. Protoplasts are gradually re-adjusted to iso-osmotic conditions and are embedded in molten agar growth media. Over the course of a few (e.g. four) days without antibiotic selection, protoplasts regenerate their cell walls and regrow into protonemal filaments. Regenerated protonema can then be moved onto antiobiotic selection in order to isolate transformants.

Plant tissue.

Chloronema can be grown on BCD+NH_4-tartrate plates overlaid with sterile cellophane and should be harvest 4-6 days after mechanical disruption with a blender or ultra-turrax Note that older protonema (i.e. caulonema) and gametophores are not suitable for obtaining regenerable protoplasts. Cell wall digestion can be facilitated by adjusting the media to pH 5.8 and omitting $CaCl_2$.

Synthesis and Preparation of DNA Knockout Constructs
1. Synthesize knockout constructs by PCR using Takara Primestar GXL DNA polymerase
2. Analyze PCR product by agarose gel electrophoresis and ensure that there is a single product of the correct size
3. Purify and concentrate PCR product using Invitrogen PureLink PCR Purification Kit - Use supplied binding Buffer B1. - Apply 2-4 times the recommended PCR product volume to each column in order to achieved desired concentration - Elute using Elution Buffer E1 (10 mM Tris-HCL, pH 8.5)
4. Measure DNA concentration by Nanodrop and adjust to 500 ng / µL

Stock Solution Formulas	**Description**	**pH**
	0.8% Driselase solution in 8% mannitol	-
	1.0 M $Ca(NO_3)_2$	-
	1.0 M $MgCl_2$	-
	1.0 M Tris-HCl	8.0
	1.0 % methyl ethyl sulfate (MES)	5.6
	8.0 % mannitol dissolved in 10% BCD+NH_4-tartrate	5.8
	8.5 % mannitol dissolved in 10% BCD+NH_4-tartrate	5.8
	10.3 % mannitol dissolved in 10% BCD+NH_4-tartrate	5.8

Fresh Solution Formulas		**Description**	**Amount**
	PEG Solution	Molten PEG	4 g
		1 M Tris, pH 8.0	100 mL
		8.5% Mannitol	100 mL
	MMM Solution	10.3% Mannitol	17.7 mL
		1.0% MES	2 mL
		1 M MgCl2	300 mL

Protoplast Transformation Protocol	
1. Harvest 5-6 day old protonemal tissue	
2. Incubate in 0.8% Driselase solution for 30 - 60 minutes (visually check for digestion)	
3. Filter protoplasts, first through 100 µM mesh filter, then 70 uM mesh filter	
4. Spin down protoplasts for 5 minutes (mins) at approximately 1,000 rotations per minute (RPM) using swinging bucket centrifuge	
5. Wash twice with 8% mannitol, resuspend in 10 mLs 8% mannitol	
6. Quantify protoplast density using haemocytometer	
7. Spin down protoplasts, resuspend in MMM (see Section 4) solution at 1.6 x 10^6 protoplasts / mL	
8. Prepare transformation mix	
PEG Solution	300 µL
Protoplasts	300 µL
DNA (500-1000 ng / µL)	40 µL
9. Heat shock at 45º C for 5 minutes	
10. Cool at RT for 5 minutes	
11. Add 500 uL 8% mannitol, wait 3 mins	
12. Add 500 uL 8% mannitol, wait 3 mins	
13. Add 500 uL 8% mannitol, wait 3 mins	
14. Add 1 mL 8% mannitol, wait 3 mins	
15. Add 1 mL 8% mannitol, wait 3 mins	
16. Add 2 mL 8% mannitol, wait 3 mins	
17. Add 3 mL 8% mannitol, wait 3 mins	
18. Add 2 mL 8% mannitol, wait 3 mins	
19. Spin down protoplasts, remove all but ~1 mL of supernatant, gently resuspend	
20. Add 10 mL molten PRMT media (see Section 4), mix gently	
21. Apply protoplast-media suspension onto three cellophane-overlaid plates	
22. On day 5 post-transformation, move to selection media (BN pH 5.8, Hi-Gel Agar)	

Notes on selection & regeneration.

After five days, protoplasts should regenerate into protonemal filaments and are ready to transfer to antibiotic selection media. Within a couple weeks, individual foci can be observed by eye. Foci should be removed from antibiotic selection and either (a) monitored for retention of a visual selection marker such as RFP or (b) placed back on antibiotic selection following a period of a week or more without selection. Stably transformed lines can be analyzed by PCR.

Section 3: Biolistic transformation procedure for random transgene integration.

Overview.

Compared to protoplast transformation, biolistic transformation offers advantages in terms of speed, flexibility, scalability, and reliability. However, as previously mentioned, biolistic transformation does not appear to stimulate gene targeting events. A variety of cell types – including chroronema, caulonema, spores and brood cells – can be rapidly transformed using the procedures described here. For most purposes, chloronemal cells serve as the most facile targets for transformation, however the capacity to transform other cell types could prove extremely useful in developing procedures for transformations of other bryophytes. Moss tissue is cultured on solid media overlain with cellophane, which facilitates transfer onto selective media. Tissue can be moved onto antibiotic selection as early as the next day following biolistic transformation. In lieu of detrimental growth phenotypes, transformed colonies can be isolated in roughly two weeks.

Plant tissue.

Chloronema can be grown on BCD+NH_4-tartrate plates overlaid with sterile cellophane. Caulonema formation can be promoted by growth on BCD plates containing auxins or lacking BCD+NH_4-tartrate. Brood cell (syn. brachycyte) formation can be induced by addition of 100 µM ABA to BCD+NH_4-tartrate media. Gametangia formation can be elicited in *Physcomitrella* by specific growth conditions (described by Cove, 2005). Briefly, gametophores are grown in minimal media (e.g. BCD media without the addition of diammonium tartrate, as described further in Section 4), day length (i.e. light exposure) is shortened from approximately 18 hours to 8 hours, and the temperature is lowered from approximately 25 to 16 degrees Celsius. After formation of gametangia, fertilization can be facilitated by irrigation of gametophores with sterile water. Mature spore capsules turn from green to brown and can be collected using forceps, taking care to avoid puncturing capsules. Non-sterile spore capsules can be easily sterilized by washing with 10% bleach, 0.1% Triton X-100 followed by 70% EtOH, then rinsing six times with sterile water. Capsules can be crushed using a sterile pestle, and spores can be isolated using an appropriately sized (e.g. 70 µM) filter and quantified using a haemocytometer.

Preparation of Plasmid DNA for Biolistic Delivery
1. Extract plasmid DNA using Qiagen Hi-speed Midiprep Kit - Grow 150 mL culture of bacteria containing desired plasmid - Follow manufacturers recommendation to extract using the Qiagen Hi-speed Midiprep Kit - Elute with 1 mL Buffer EB (10 mM Tris-HCl, pH 8.5), expect to recover ~750 µL
2. Concentrate plasmid DNA using Invitrogen PureLink PCR Purification Kit - Resuspend using 50 µL of Elution Buffer E1 (10 mM Tris-HCl, pH 8.5)
3. Measure DNA concentration using nanodrop, and adjust to 1100 ng / µL

Consumable Materials for Biolistic Transformation
Bio-Rad Rupture disks (1100 PSI)
Seashell Technology LLC DNAdel™ S1000d 1 µM gold particles
100% EtOH, -20º C
[Cellophanes] or (for spores) Whatman GF/C glass microfiber filters, 70 mM diameter

Biolistic Transformation Protocol
1. Sterilize the gene gun and all component, using sterile technique
2. Precipitate DNA onto gold particles following manufacturers recommendations - Vortex particles at max velocity for > 5 minutes - Mix binding buffer, gold particles, and DNA at specified ratios (e.g. see below) Gold particles 45 µL Binding buffer 30 µL DNA (1100 ng/uL) 7.5 µL - Vortex vigorously - Add equal volume of precipitation buffer (e.g. 82.5 µL) dropwise while vortexing - Vortex vigorously, allow to sit for 3 minutes - Centrifuge at 10,000 rpm for 10 seconds using microfuge - Remove supernatant, wash particles with 500 µL 100% EtOH - Centrifuge at 10,000 rpm for 10 seconds using microfuge - Remove supernatant, resuspend in desired volume of 100% EtOH (e.g. 90 µL)
3. Apply 10 uL of gold particles per macrocarrier
4. Shoot each plate one time
5. Allow protonema to recover 1 - 2 days in growth chamber
6. Move protonema onto fresh selection media (e.g. 50 µg / mL Hygromycin B or G418)
7. After 1 - 2 weeks, move surviving colonies onto fresh selection media without cellophanes

Section 4: Media Formulation.

Overview.

This section provides instructions for formulating *Physcomitrella* growth media. Formulas are based on previously described growth media with minor modifications. Storage of liquid stock solutions for subsequent mixing helps to prevent precipitation or contamination.

Stock Solutions		
Name	**Description**	**Amount**
Solution B	$MgSO_4 \cdot 7\ H2O$	25 g
Solution C	KH_2PO_4	25 g
	* Adjust to pH 6.5 using 10 M KOH	
Solution DK	KNO_3	101 g
Solution DF	Tartrate Buffer, pH 4.0	10 mM
	$FeSO_4 \cdot 7\ H_2O$	1.25 g
	* Separation of solution D into two mixes and buffering each to acidic pH greatly reduces precipitate formation	
Trace Element Solution	H_3BO_3	614 mg
	$CuSO_4 \cdot 5\ H_20$	55 mg
	KBr	28 mg
	$Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24\ H_20$	55 mg
	$MnCl_2 \cdot 4\ H_20$	389 mg
	$ZnSO_4 \cdot 7\ H_20$	55 mg
	LiCl	28 mg
	$CoCl_2 \cdot 6\ H_20$	55 mg
	KI	28 mg
	$SnCl_2 \cdot 2\ H_20$	28 mg
1 M $CaCl_2$ Solution	Autoclave and store under sterile conditions	

Growth Media Recipes (for 1 Liter each)		
Media Type	**Component**	**Amount**
BN media	Solution B	10 mL
	Solution C	10 mL
	Solution DK	10 mL
	Solution DF	10 mL
	500 mM NH_4-tartrate [check]	10 mL
	Trace Element Solution	1 mL
	(post-autoclave) 1 M $CaCl_2$ Solution	1 mL
	(for solid media) Phytoblend [specifics]	8 g
	(or for regenerating protoplasts) Sigma Hi-Gel	
Protoplast regeneration media, bottom layer (PRMB)	Solution B	10 mL
	Solution C	10 mL
	Solution DK	10 mL
	Solution DF	10 mL
	500 mM NH_4-tartrate	10 mL
	Trace Element Solution	1 mL
	D-mannitol	60 g
	* Adjust to pH 5.8 using KOH or HCl	
	(for solid media) Sigma Hi-Gel Agar	7 g
	(post-autoclave) 1 M $CaCl_2$ Solution	10 mL

Growth Media Recipes		
Media Type	**Component**	**Amount**
Protoplast regeneration media, bottom layer (PRMB)	Solution B	10 mL
	Solution C	10 mL
	Solution DK	10 mL
	Solution DF	10 mL
	500 mM NH_4-tartrate	10 mL
	Trace Element Solution	1 mL
	D-mannitol	60 g
	* Adjust to pH 5.8 using KOH or HCl	
	(for solid media) Sigma Hi-Gel Agar	7 g
	(post-autoclave) 1 M $CaCl_2$ Solution	10 mL
Protoplast regeneration media, top layer (PRMT)	Solution B	10 mL
	Solution C	10 mL
	Solution DK	10 mL
	Solution DF	10 mL
	500 mM $(NH_4)_2$-tartrate	10 mL
	Trace Element Solution	1 mL
	D-mannitol	80 g
	Adjust to pH 5.8 using KOH or HCl	
	(for solid media) Sigma Hi-Gel Agar	4 g
	(post-autoclave) 1 M $CaCl_2$ Solution	10 mL

Sources Cited.

Ashton N.W., Champagne C.E.M., Weiler T., & Verkoczy L.K. (2000). The bryophyte *Physcomitrella patens* replicates extrachromosomal transgenic elements. *New Phytologist* 146, 391-402.

Bopp (2000) 50 years of the moss story. *Progress in Botany* 61, 3-34.

Cho S.-H., Chung Y.-S., Cho S.-K., Rim Y.-W., and Shin J.-S. (1999). Particle bombardment mediated transformation and GFP expression in the moss *Physcomitrella patens*. *Molecules and Cells* 9, 14-19.

Cove D.J., Schild A., Ashton N. W., and Hartmann E. (1978). Genetic and Physiological Studies of the Effect of Light on the Development of the moss, *Physcomitrella patens*. *Photochemistry and Photobiology* 27, 249-254.

Cove D. (2005). The moss *Physcomitrella patens*. *Annual Review of Genetics* 39, 339-358.

Cove D.J., Perroud P.F., Charron A.J., Mcdaniel S.F., Khandelwal A., and Quatrano R.S. (2009a). Transformation of moss *Physcomitrella patens* gametophytes using a biolistic projectile delivery system. *Cold Spring Harb Protocols* 2009:

Cove D.J., Perroud P.F., Charron A.J., McDaniel S.F., Khandelwal A., and Quatrano R.S. (2009b). Transformation of the moss *Physcomitrella patens* using direct DNA uptake by protoplasts. *Cold Spring Harb Protocols* 2009:

Cove D.J., Perroud P.F., Charron A.J., Mcdaniel S.F., Khandelwal A., and Quatrano R.S. (2009c). Transformation of the moss *Physcomitrella patens* using T-DNA mutagenesis. *Cold Spring Harb Protocols* 2009.

Li L.-H., Yang J., Qiu H.L., & Liu Y.Y. (2010). Genetic transformation of *Physcomitrella patens* mediated by *Agrobacterium tumefaciens*. African Journal of Biotechnology 9, 3719-3725.

Li X., Zhang D., Li H., Gao B., Yang H, Zhang Y., amd Wood A.J. (2015). Stable Characterization of reference genes for RT-qPCR in the desert moss *Syntrichia caninervis* in response to abiotic stress and desiccation/rehydration. *Frontiers in Plant Science* 6, 38.

Mann D.G.J., Lafayette P.R., Abercrombie L. L., King Z.R., Mazarei M., Halter M.C., Poovaiah C.R., Baxter H., Shen H., Dixon R.A., Parrott W.A., and Neal Stewart Jr. C. (2012). Gateway-compatible vectors for high-throughput gene functional analysis in

switchgrass (*Panicum virgatum* L.) and other monocot species. *Plant Biotechnology Journal* 10, 226-236.

Muthukumar B., Joyce B.L., Elless M.P., and Neal Stewart Jr. C. (2013). Stable Transformation of Ferns Using Spores as Targets: *Pteris vittata* and *Ceratopteris thalictroides*. *Plant Physiology* 163, 648-658.

Park S.-Y., Vaghchhipawala Z., Vasudevan B., Lee L.-Y., Shen Y., Singer K., Waterworth W.M., Zhang Z.J., West C.E., Mysore K.S., & Gelvin S.B. (2015). *Agrobacterium* T-DNA integration into the plant genome can occur without the activity of key non-homologous end-joining proteins. *The Plant Journal* 81, 934-946.

Rensing S.A., Lang D., Zimmer A.D., Terry A., Salamov A., Shapiro H., …, & Boore J.L. (2008). The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* 319, 64-69.

Sawahel W., Onde S., Knight C., & Cove D. (1992). Transfer of foreign DNA into *Physcomitrella patens* protonemal tissue by using the gene gun. *Plant Molecular Biology Reporter* 10, 314-315.

Schaefer D.G. & Zrÿd J. (1997). Efficient gene targeting in the moss *Physcomitrella patens*. *The Plant Journal* 11: 1195-1206.

Šmídková M., Holá M., & Angelis K.J. (2010). Efficient biolistic transformation of the moss *Physcomitrella patens*. *Biologia Plantarum* 54, 777-780.

Szovenyi P., Perroud P.F., Symeonidi A., Stevenson S., Quatrano R.S., Rensing S.A., Cuming A.C., & Mcdaniel S.F. (2015). *De novo* assembly and comparative analysis of the *Ceratodon purpureus* transcriptome. *Molecular Ecology Resources* 15: 203-215.

Szovenyi P., Ricca M., Hock Z., Shaw J.A., Shimizu K.K., & Wagner A. (2013). Selection is no more efficient in haploid than in diploid life stages of an angiosperm and a moss. *Molecular Biology & Evolution* 30, 1929-1939.

Wendeler E., Zobell O., Chrost B., & Reiss B. (2015). Recombination products suggest the frequent occurrence of aberrant gene replacement in the moss *Physcomitrella patens*. *The Plant Journal* 81, 548-558.

Weston D.J., Timm C.M., Walker A.P., Gu L., Muchero W., Schmutz J., Shaw A.J., Tuskan G.A., Warren J.M., & Wullschleger S.D. (2014). *Sphagnum* physiology in the context of changing climate: emergent influences of genomics, modelling and host–microbiome interactions on understanding ecosystem function. *Plant, Cell, & Environment* (early view online).

von Wettstein, F. (1932). Genetik. In: *Manual of Bryology* (Ed. F. Verdoorn). Martinus Nijhoff, The Hague, pp 233-273.

Chapter 5: Functional characterization of a calcium-regulated protein kinase and target transcription factor in *Physcomitrella* using a gain-of-function approach.

Preface.

In the previous chapter, efforts to improve techniques for *Physcomitrella* genetic transformation were described. In the following chapter, these techniques were employed to study the function of a calcium-regulated protein kinase. Computational approaches suggested that this protein kinase may regulate the activities of CYCLOPS, a transcription factor. This predicted regulatory interaction was corroborated using yeast two-hybrid analyses. Gain-of-function alleles of the protein kinase or transcription factor were generated by selective deletion or site-directed mutagenesis. Expression of native or gain-of-function alleles in moss cells indicated that the CCaMK-CYCLOPS signaling module positively regulates formation of brood cells, a type of stress-induced asexual propagule found in mosses. Because brood cell formation has been previously linked to abscisic acid (ABA), a stress-associated phytohormone, ABA responses were investigated in gain-of-function lines. Experiments demonstrated that expression of CCaMK-CYCLOPS gain-of-function alleles is associated with constitutive upregulation of ABA marker genes and hypersensitivity to ABA treatment. Collectively, the results indicate that the CCaMK-CYCLOPS signaling modules governs stress-induced asexual propagule formation in *Physcomitrella patens*.

Introduction.

The availability and distribution of nutrients are key environmental parameters that plants monitor closely. In response to nutrient deficits, plants commonly implement strategies to conserve resources (e.g. by slowing rates of growth) or acquire nutrients via symbiotic associations with microbes. In particular, most land plants can engage in facultative symbioses with mycorrhizal fungi, and this symbiotic relationship appears to have developed at early stages in the evolution of land plants (reviewed by Brundrett, 2002; Parniske, 2008). More recently, various lineages of plants have developed symbioses with bacteria that fix atmospheric nitrogen (N_2) in exchange for reduced carbon (Mylona *et al*., 1995). From an evolutionary perspective, the establishment of such intimate symbioses is striking. Not only do plants often host these symbionts intracellularly, the plant itself restructures its organs and cells in anticipation of microbial colonization, implicating tightly coordinated signaling processes between organisms (Oldroyd, 2013; Parniske, 2008).

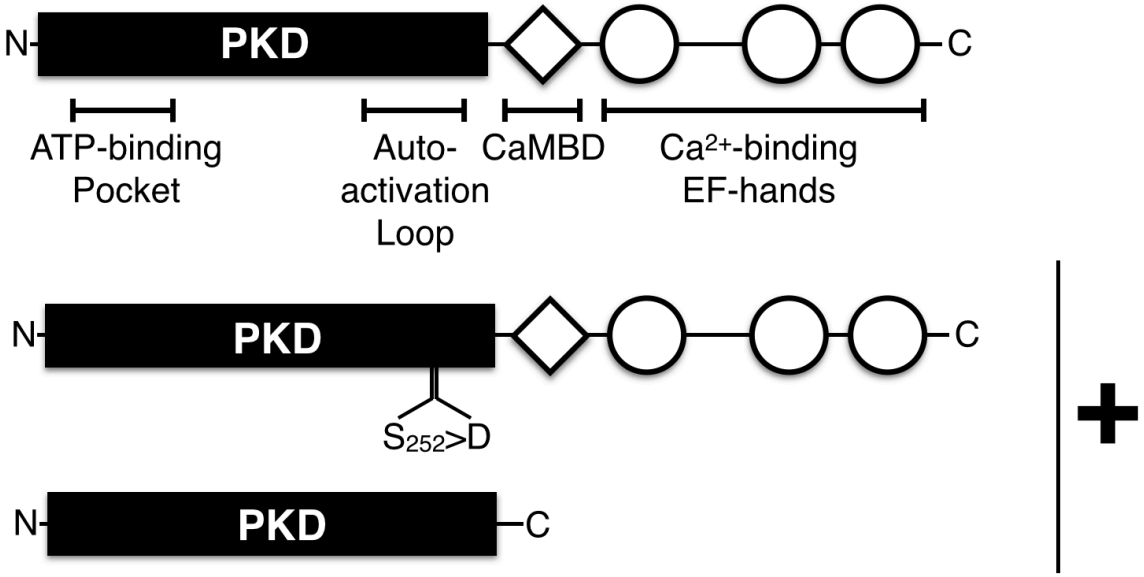


Figure 5.1: Domain architecture and gain-of-function alleles of CCaMK. The N-terminal half of CCaMK is comprised of a typical serine/threonine (S/T) protein kinase domain (PKD), which contains an ATP-binding pockete required for catalytic activity and an auto-activation loop that is autophosphorylated to activate CCaMK kinase activitiy. The C-terminal autoinhibitory region contains a predicted calmodulin-binding domain (CaMBD) and three calcium-binding EF-hands. Prior studies in legumes (Gleason *et al*. 2006; Tirichine *et al*., 2006) have identified two distinct gain-of-function alleles (+) of CCaMK. One contains a phosphomimetic substitution (S/T > aspartate, D); the other has the C-terminal half deleted.

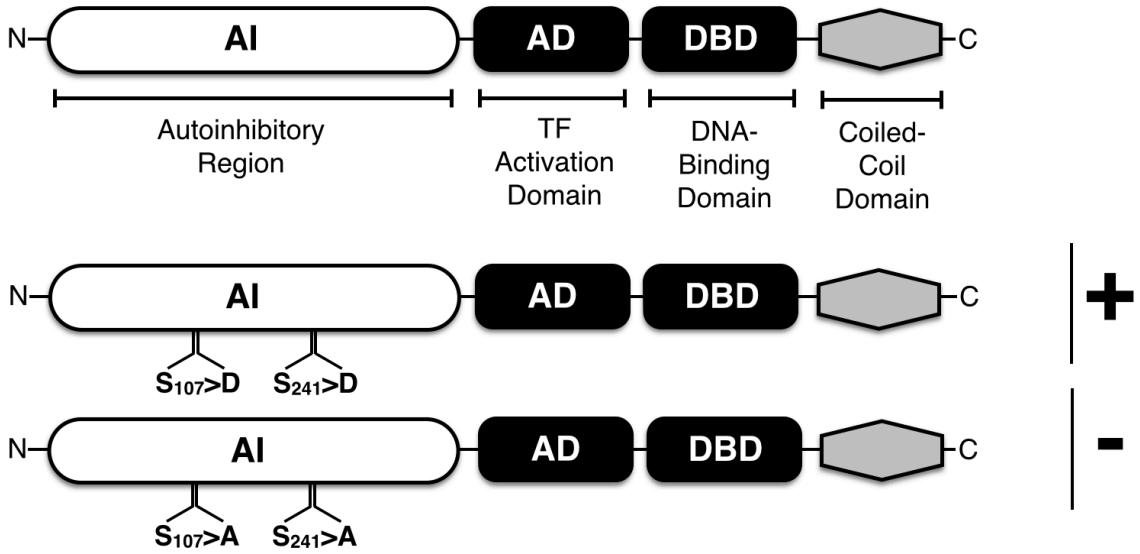


Figure 5.2: Domain architecture and gain- or loss-of-function alleles of CYCLOPS. CYCLOPS represents a novel class of transcription factor (TF) that lacks discernible homology with other TF families. CCaMK-mediated phosphosites are located in the N-terminal autoinhibitory region (AI). Two serine (S) residues have been identified that yield gain- or loss-of-function alleles when mutagenized to phosphomimetic (S > aspartate, D; +) or phosphoablative (S > alanine, A; -) substitutions, respectively.

The molecular basis for establishing symbiotic relationships between plants and other organisms has only recently begun to come to light. Surprisingly, rhizobial bacteria and mycorrhizal fungi bacteria utilize the same molecular signaling pathway, commonly referred to as the SYM pathway (Ané *et al*., 2004; den Camp *et al*., 2011; Svistoonoff *et al*., 2013). Plant-microbe associations involve signals emanating from both plants (Bécard *et al*., 1990) and microbes (Maillet *et al*., 2011). Mycorrhizal fungi and rhizobial bacteria emit signals termed Myc and Nod factors, respectively, that share similar chemical structures (Brelles-Marino & Ané, 2008). These signaling factors bind to cognate receptor-like kinases (RLKs) (Endre *et al*., 2002; Limpens *et al*., 2003) and lead to calcium spiking patterns in the nucleus of the host (Oldroyd & Downie, 2006; Capoen *et al*., 2011; Chabaud *et al*., 2011) that are decoded by a calcium/calmodulin-dependent protein kinase (CCaMK) (Lévy *et al*., 2004). A loss-of-function mutant defective in CCaMK was originally identified through genetic screening as *doesn't make infections 3* (DMI3), and these mutants showed blockage of colonization by rhizobia or mycorrhiza. CCaMK gain-of-function alleles with the C-terminal autoinhibitory region deleted (Gleason *et al*., 2006) or point mutations in the kinase autoactivation loop (Tirichine *et al*., 2006) render the kinase hyperactive (Figure 5.1). Expression of either form of gain-of-function allele in roots is sufficient to induce autonodulation (i.e. nodule formation in the absence of rhizobia).

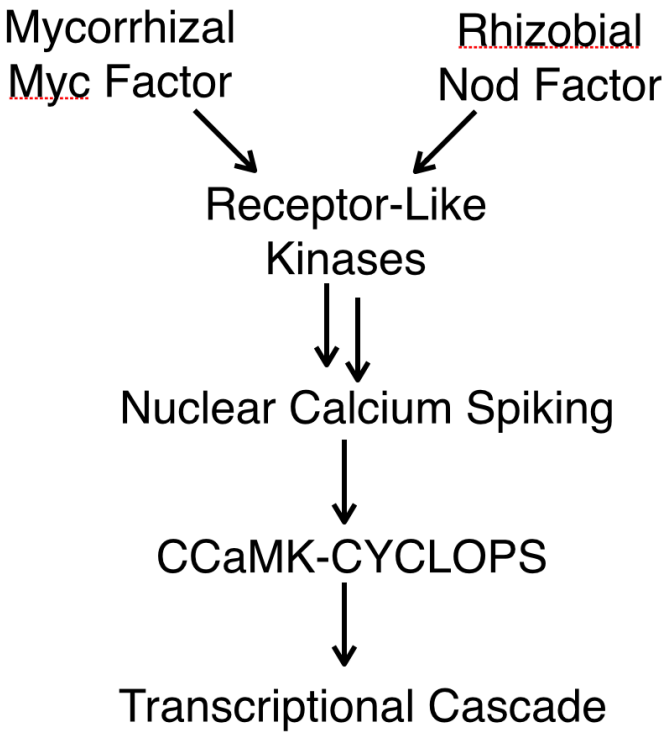


Figure 5.3: Diagram showing key components and events in the plant-microbe symbiosis. In legumes, signals emanating from mycorrhiza or rhizobia are detected by receptor-like kinases (RLKs). Although intermediary steps are not fully characterized, RLK activation ultimately leads to calcium spiking in the perinuclear region. The calcium oscillation are decoded by CCaMK, which in turn regulates the activities of CYCLOPS. Activation of CYCLOPS lead to a transcriptional cascade that mediates symbiotic associations.

CYCLOPS, also known as INTERACTING PARTNER OF DMI3 (IPD3), is a novel type of transcription factor that was identified through yeast two-hybrid screens for interactors with legume CCaMK. CYCLOPS has been shown to act as a phosphorylation substrate of CCaMK *in vitro*, and two phosphosites that are necessary and sufficient for CYCLOPS activation have been pinpointed (Yano *et al*., 2008; Singh *et al*., 2014). Phosphomimetic substitutions at these two sites lead to constitutive activation of the transcription factor (Figure 5.2), and expression of a dual-phosphomimetic form of CYCLOPS in roots causes autonodulation (Singh *et al*., 2014). Hence, current models suggest that CCaMK-CYCLOPS act as a signaling module that decodes nuclear calcium signals and initiates a transcriptional cascade that coordinates plant-microbe symbioses (Figure 5.3).

Although the common ancestor of extant land plants likely exhibited mycorrhizal associations and early-diverging plants like the liverwort *Marchantia* show mycorrhizal associations (Kenrick & Crane, 1997; Brundrett, 2002; Wang et al., 2006), some plant lineages have apparently lost the ability to form mycorrhizal symbioses. Notably, the model plant Arabidopsis, a member of the mustard family, is unusual compared to most plants in that it does not accommodate mycorrhiza (Wang *et al*., 2006; Veiga *et al*., 2013). Interestingly, the genomes of Arabidopsis and other non-mycorrhizal angiosperms plants have apparently lost the genes encoding CCaMK, CYCLOPS, and other components of the SYM pathway (Wang *et al*., 2010; Delaux *et al*., 2014). The observed pattern of gene loss was previously interpreted as evidence for a dedicated function of CCaMK-CYCLOPS in mycorrhizal associations; however, among early-

diverging plants, the moss *Physcomitrella* has unexpectedly retained the CCaMK-CYCLOPS module (Read *et al*., 2000; Wang *et al*., 2010; Proust *et al*., 2011). Unlike other early-diverging, bryopsids or "true mosses" — the group to which *Physcomitrella* belongs, have been generally considered mycorrhizal-incompetent, and efforts to establish symbiotic associations among *Physcomitrella* and mycorrhizal fungi in culture have been unsuccessful (personal communication J.M. Ané, University of Wisconsin – Madison). Moreover, CCaMKs from mycorrhizal-competent early-diverging plants can functionally complement legume *ccamk* loss-of-function, whereas CCaMKs from *Physcomitrella* or other non-mycorrhizal mosses cannot. This observation piqued our interest in possible functions of CCaMK-CYCLOPS aside from symbiosis and led us to pursue functional genetic analysis of the CCaMK-CYCLOPS in *Physcomitrella*.

Materials & Methods:

Plant growth conditions and nucleic acid isolation.

Physcomitrella patens Gransden 2004 strain was grown under 16 hours day / 8 hours night conditions with approximately 100 microeinsteins light provided by cool white fluorescent bulbs. Growth media are described in Chapter 4. Nucleic acid extractions were performed as described previously (Chapter 3).

Cloning and site-directed mutagenesis.

Physcomitrella RNA was reverse transcribed using Quantitect Reverse Transcriptase (Qiagen) containing a proprietary mix of random and oligo d(T) primers. Site directed mutagenesis was performed by whole-plasmid amplification using anti-complementary primers and Phusion (Thermo Scientific) or Primestar GXL (Takara) DNA Polymerase. Amplicons were digested overnight with Dpn I (New England Biolabs) and transformed into chemically competent *E. coli*.

Plant transformation.

Biolistic transformation was performed using Seashell Technology 1 micron gold particles and a Bio-Rad PDS-1000/He gene gun system following the procedures described in Chapter 4. Stable transformants were verified by red fluorescent protein (RFP) signal.

Microscopy.

Micrographs were taken using a Zeiss Axioimager M1 microscope (Carl Zeiss Inc.). Manually taken z-stacks were assembled using Helicon Focus software (HeliconSoft).

Gene expression analysis.

Gene expression was analyzed by qRT-PCR. RNA was reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen) following manufacturers recommendation. Amplification was performed using a DNA-Engine Opticon system for continuous fluorescence detection (MJ Research) and iTaq universal SYBR green supermix (Bio-Rad). All analyses were performed in quadruplicate. The comparative cycle threshold ($\partial/\partial C_t$) method was used to compare expression levels. ABA-inducible marker genes and three stably expressed reference genes were selected from previous studies (Shinde *et al*., 2012; Shinde *et al*., 2013; Le Bail *et al*., 2013).

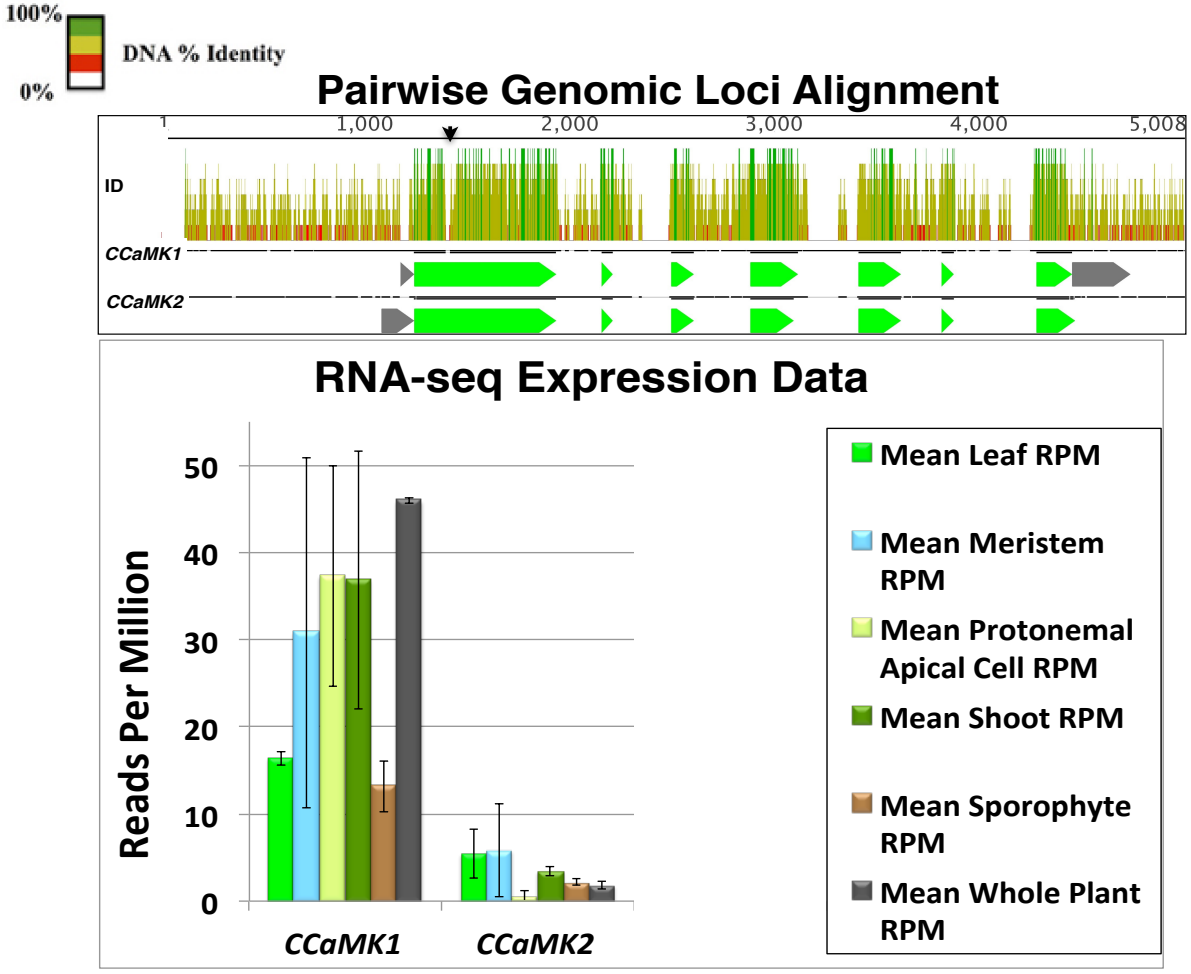
Yeast two-hybrid analysis.

Yeast two-hybrid assays were performed using the Clontech matchmaker system based on a split GAL4 transcription factor. Interactions were assayed on growth media lacking histidine (H) and adenine (A) as auxotrophic markers, as described previously (Chapter 3).

Results & Discussion:

Previously, it was reported that *Physcomitrella*, like other early-diverging land plants, contained loci encoding homologs of CCaMK, CYCLOPS, and other SYM pathway components (Wang *et al*., 2010). Using bioinformatic approaches, we identified two loci in the *Physcomitrella* genome that encode CCaMKs with 72% identity at the amino acid level and used available transcriptomic data to analyze their expression patterns (Figure 5.4). We took advantage a previous investigation that used laser-assisted microdissection and transcriptomics (RNA-seq) to analyze expression patterns in *Physcomitrella* tissues (Frank & Scanlon, 2015). One locus, which we named *CCaMK1*, was expressed at higher levels than its paralog, which we named *CCaMK2*, in all analyzed tissues. We cloned transcripts from each locus and verified their predicted-exon intron structures.

Figure 5.4: Genomic loci encoding CCaMKs in *Physcomitrella*. (Top) *Physcomitrella CCaMK1* and *CCaMK2* share an identical exon-intron structure and show a great degree of conservation in their exonic regions, except for part of the first exon (arrowhead). (Bottom) Laser-assisted microdissection coupled with RNA-seq (Frank & Scanlon, 2015) indicated that *CCaMK1* is predominantly expressed in all tissues, which is consistent RT-PCR results. Transcript levels are provided in reads per million (RPM).



Because previous studies in legumes identified CCaMK gain-of-function alleles that led to constitutive kinase activation and visibly obvious phenotypes *in planta*, we implemented a similar strategy with the aim to functionally characterize *Physcomitrella* CCaMK1. We cloned cDNAs encoding either full-length CCaMK1 ($CCaMK1_{FL}$)or the kinase domain of CCaMK1 without the C-terminal autoinhibitory region ($CCaMK1_{KD}$) and subcloned these constructs into the pANIC5A vector (Figure 5.5) for expression in *Physcomitrella* cells driven by the Zea mays *UBIQUITIN* 1 (ZmUBI1) promoter (Mann *et al*., 2012), which has been shown to confer strong expression in *Physcomitrella* (Bezanilla *et al*., 2003). The resulting plasmids were delivered into *Physcomitrella* cells

Figure 5.5: Map of pANIC5A vector used for overexpression. The pANIC5A vector contains a pair of gateway cloning sites (R1, R2) flanking the gateway cloning cassette (GW) located to the 3' end of the maize ubiquitin 1 promoter (ZmUBI1). The vector contains a hygromycin resistance gene (HPH) driven by the rice actin 1 promoter (OsAct1) for chemical selection and a red fluorescent protein (RFP) gene driven by the switchgrass (*Panicum virgatum*) ubiquitin 1 promoter (PvUbi1). Introns are indicate by dashed wedges.

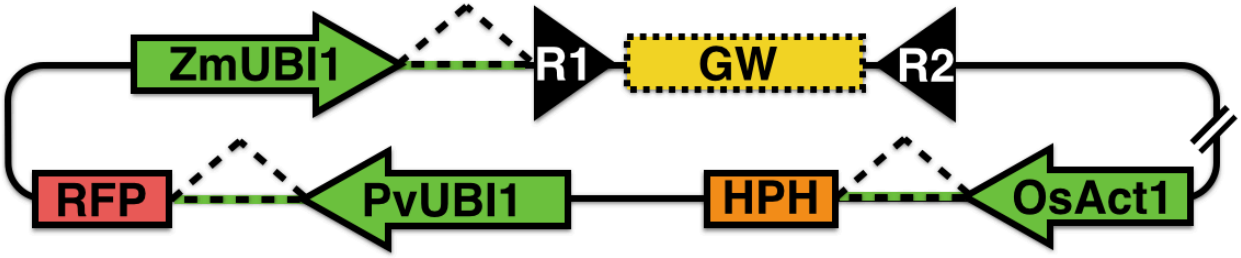
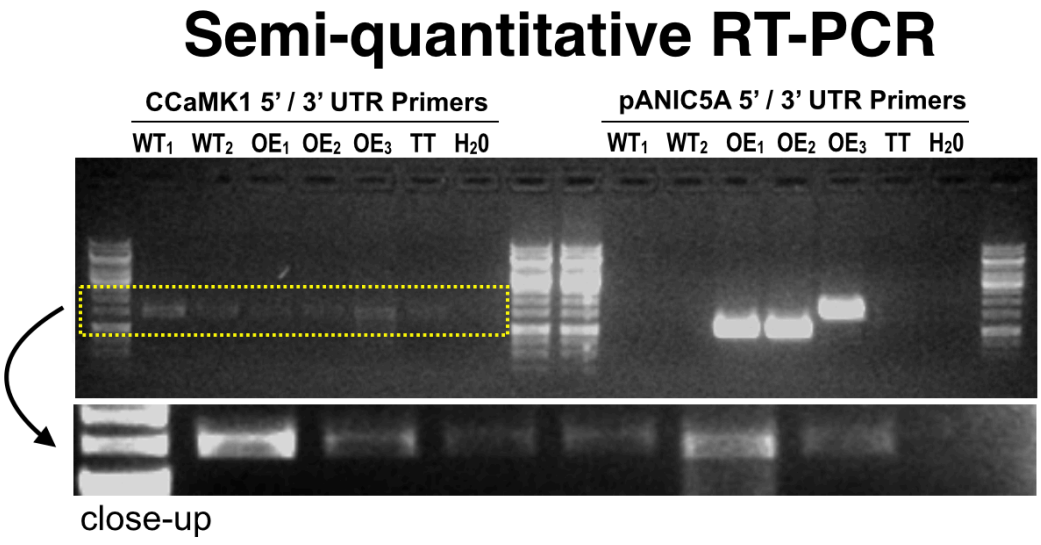


Figure 5.6: RT-PCR showing expression of transgenes driven by the ZmUBI1 promoter in the pANIC5A vector. Oligonucleotide primers were used that were complementary to the untranslated regions (UTR) of native *CCaMK1* or the pANIC5A vector. Two wild-type ($WT_{1,2}$) replicates, two $CCaMK1_{KD}$ overexpression lines ($OE_{1,2}$), one $CCaMK1_{FL}$ overexpression line (OE_3), one transiently transformed line (TT), and a no template / water control (H_2O) were screened.



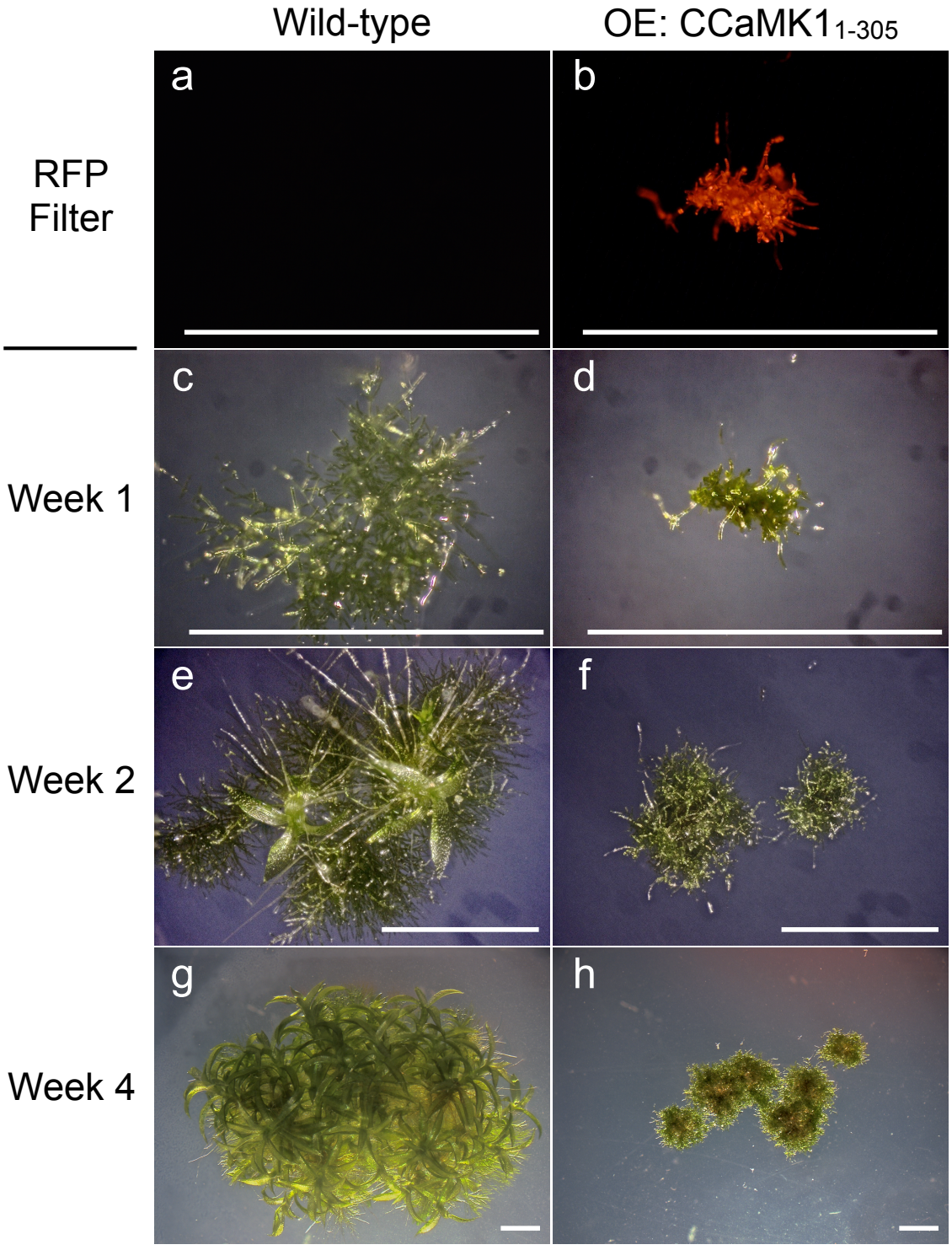


Figure 5.7: Growth phenotype of CCaMK1-KD overexpression lines. Freshly blended suspsension cultures of wild-type or CCaMK1$_{KD}$ (amino acid residues #1-305) lines were spotted onto standard moss growth media (BCD+NH_4-tartrate) and grown for the indicated period of time. Note that CCaMK1KD lines form denser colonies than wild-type and fail to develop gametophores. Scale bars indicate 2 millimeters (mm).

by particle bombardment (described in Chapter 4), and stable transgenic lines were generated by extended culture on antibiotic selection media and visual monitoring of a RFP plasmid marker. Stably transformed lines expressing $CCaMK1_{KD}$ (Figure 5.6) showed retarded growth and formed colonies with several obvious deviations from wild-type colonies (Figure 5.7).

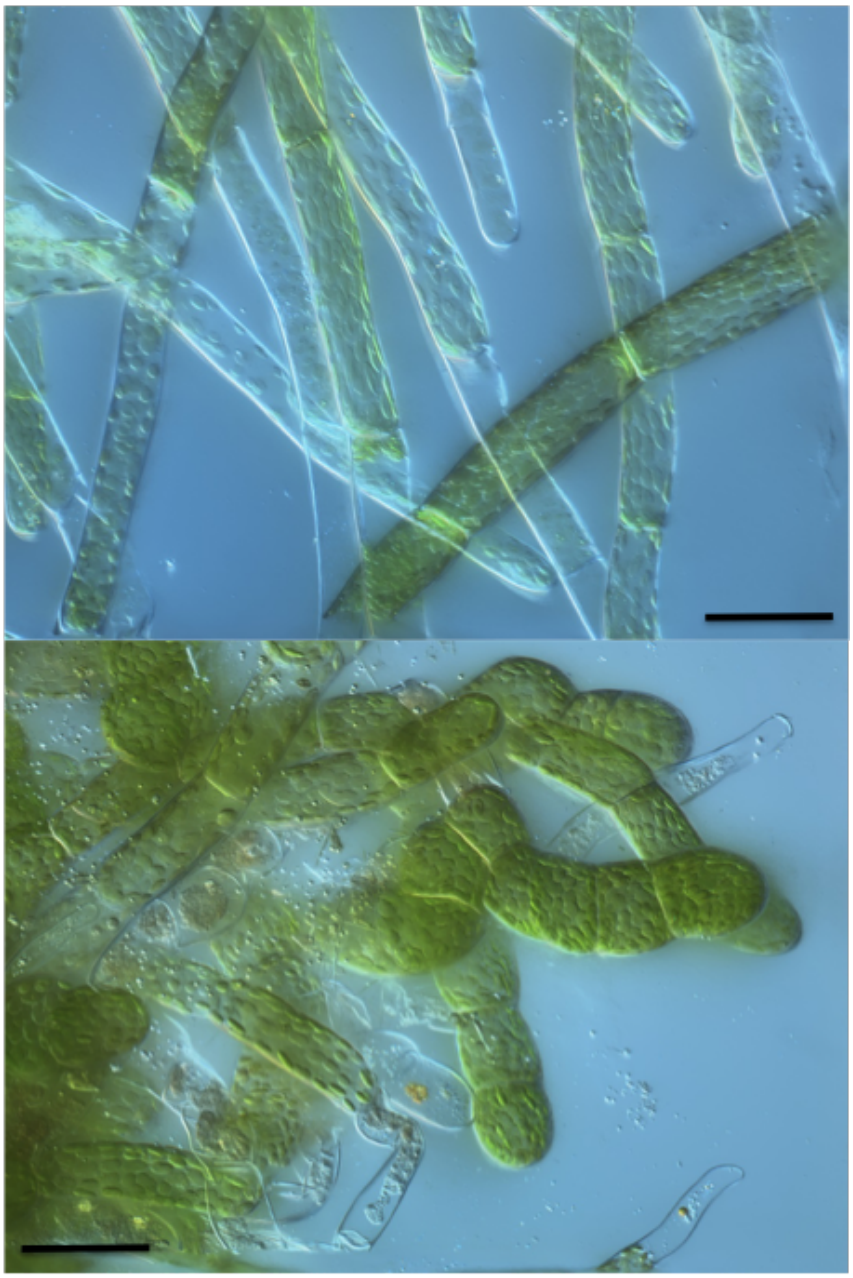
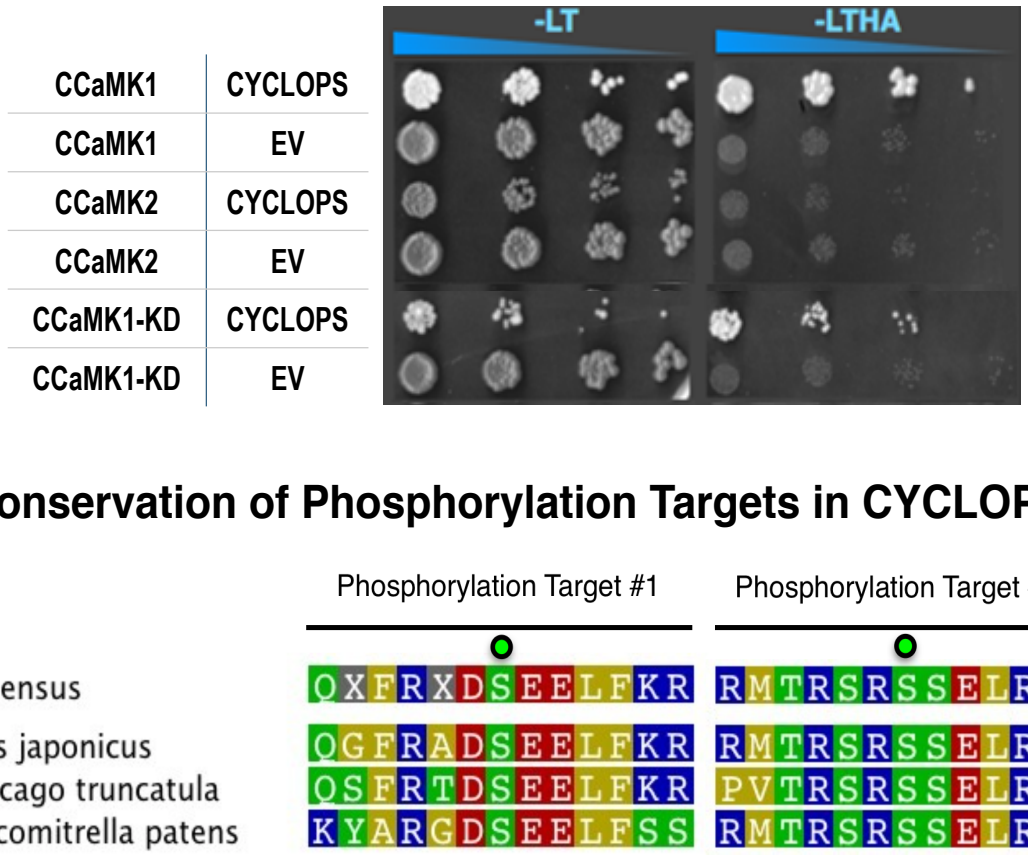


Figure 5.8: Growth phenotype of CCaMK1-FL or CCaMK1-KD overexpression lines. (Top) Overexpression of $CCaMK1_{FL}$ using the pANIC5A vector does not lead to brood cell formation; lines produce elongate protonemal cells. (Bottom) Overexpression of $CCaMK1_{KD}$ is associated with formation of nearly spherical brood cells with distinctive cell wall thickenings. Micrographs taken using differential interference contrast (DIC). Scale bars indicate 50 microns.

CCaMK1$_{KD}$ gain-of-function lines formed colonies that grew more densely than wild-type and failed to form gametophores, even after extended culture. Closer inspection of cells expressing CCaMK1$_{KD}$ revealed gross morphological aberrations compared to wild-type cells or lines expressing full-length CCaMK1$_{FL}$ (Figure 5.8). Rather than developing elongate filamentous cells seen in wild-type or CCaMK1$_{FL}$ lines, CCaMK1$_{KD}$ lines formed nearly spherical cells with distinctive thickenings in the cell wall that were prominantly visible under differential interference contrast (DIC) microscopy. Whereas CCaMK1KD lines do not develop gametophores (see Figure 5.7), CCaMK1$_{FL}$ lines are capable of forming gametophores, but these are dwarfed (data not shown).

Figure 5.9: Regulation of CYCLOPS by CCaMK appears to be conserved in *Physcomitrella*. (Top) Yeast 2-hybrid analyses indicated that *Physcomitrella* CCaMK1, but not CCaMK2, physically interacted with CYCLOPS and that the kinase domain of CCaMK1 (CCaMK1-KD) was sufficient for interaction. The empty vector controls (EV) did not show interactions. Ten-fold serial growth dilutions were performed, starting with culutures adjusted to optical density at 600 nanometers (OD_{600}) = 0.05. (Bottom) The two key CYCLOPS residues subject to phosphoregulation by CCaMK in legumes are conserved in *Physcomitrella*.



Because CYCLOPS is a functional partner of CCaMK in legumes and homologs co-present or co-absent with CCaMKs in sequenced plant genomes, we tested whether PpCCaMK1 and PpCYCLOPS would show physical interactions in yeast cells by two-hybrid assay. *Physcomitrella* contains a single locus encoding a CYCLOPS homolog. The results showed that PpCCaMK1 and PpCYCLOPS interact with each other in a strong and specific manner (Figure 5.9 *top*). In contrast, PpCCaMK2 did not interact with PpCYCLOPS in yeast two-hybrid assays. We used a multiple sequence alignment (MSA) to identify residues in PpCYCLOPS orthologous to phosphosites in legume homologs that are necessary and sufficient for transcription factor activation (Figure 5.9 *bottom*).

We mutated the serine residues at these two sites to aspartate using site-directed mutagenesis in order to generate a dual-phosphomimetic form of PpCYCLOPS ($CYCLOPS_{S107D,S241D}$) and subcloned it into the pANIC5A vector for expression in *Physcomitrella*. *Physcomitrella* lines transformed with dual-phosphomimetic CYCLOPS showed a similar phenotype to $CCaMK1_{KD}$ lines, however expression of native CYCLOPS using the same vector did not lead to any striking growth phenotype (Figure 5.10). Like CCaMK1-KD lines, $CYCLOPS_{S107D,S241D}$ lines showed drastic disparities from wild-type cells and produced cells that were globular rather than elongate in appearance. Like CCaMK1-KD lines, $CYCLOPS_{S107D,S241D}$ lines showed distinctive cell wall thickenings that could be highlighted using DIC microscopy. The absence of a growth phenotype in lines transformed with the native form of CYCLOPS supports the notion that the expression of a gain-of-function alleles of CYCLOPS *per se*, rather than mere overexpression of the gene, is required to stimulate brood cell formation.

Abscisic acid (ABA) is a phytohormone that is commonly associated with abiotic stress signaling. In mosses, ABA has been previously shown to inhibit formation of gametophore buds and to stimulate production of brood cells, a type of stress-induced asexual propagule (Goode et al., 1993; Schnepf & Reinhard, 1997). By addition of ABA to moss growth media, we showed that exogenous ABA could phenocopy CCaMK or CYCLOPS gain-of-function lines (Figure 5.11). These results further suggest that activation of the *Physcomitrella* CCaMK-CYCLOPS signaling module is associated with ABA signaling and prompted further investigation.

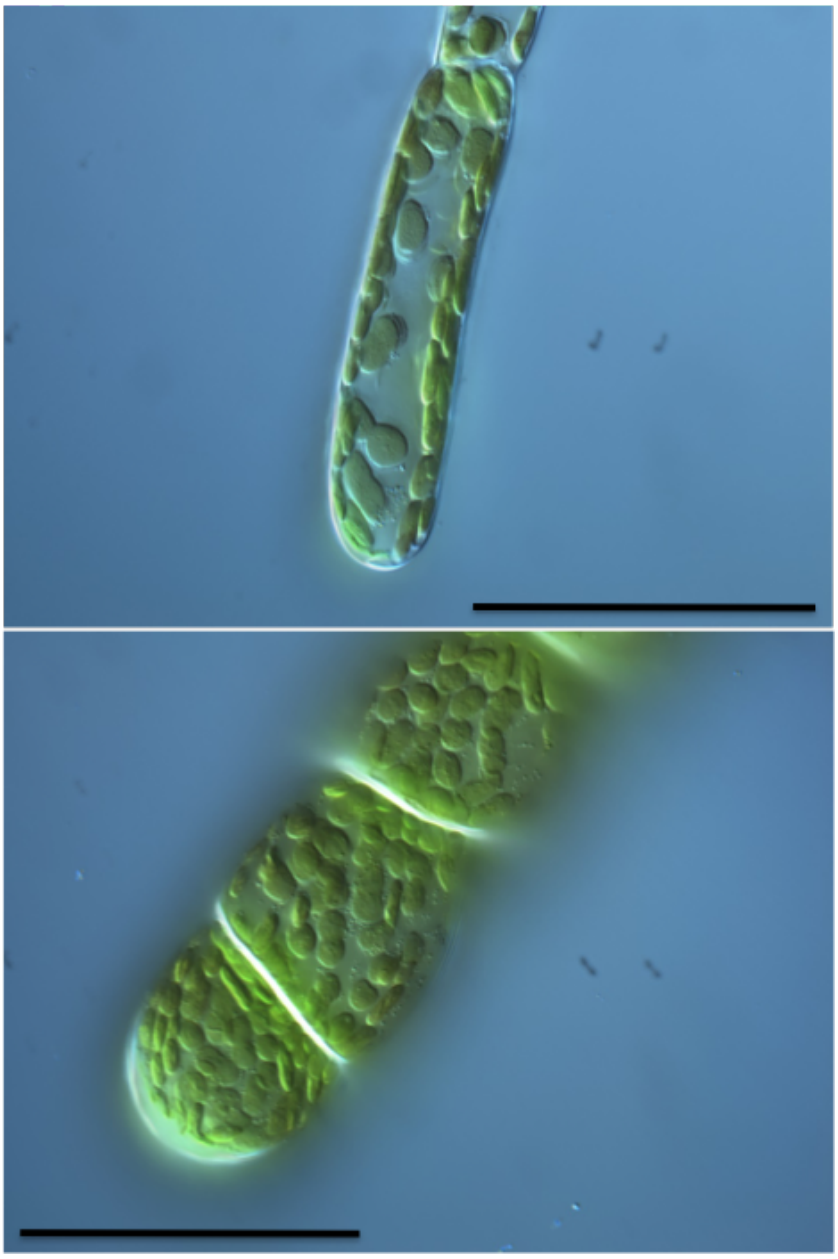


Figure 5.10: Dual-phosphomimetic substitutions in CYCLOPS recapitulate $CCaMK1_{KD}$ gain-of-function phenotype. (Top) Expresion of the native form of CYCLOPS does not lead to a discernible phenotype. (Bottom) Expression of the dual-phosphomimetic form of CYCLOPS (S107D, S241D) leads to brood cell formation. Scale bars indicate 50 microns.

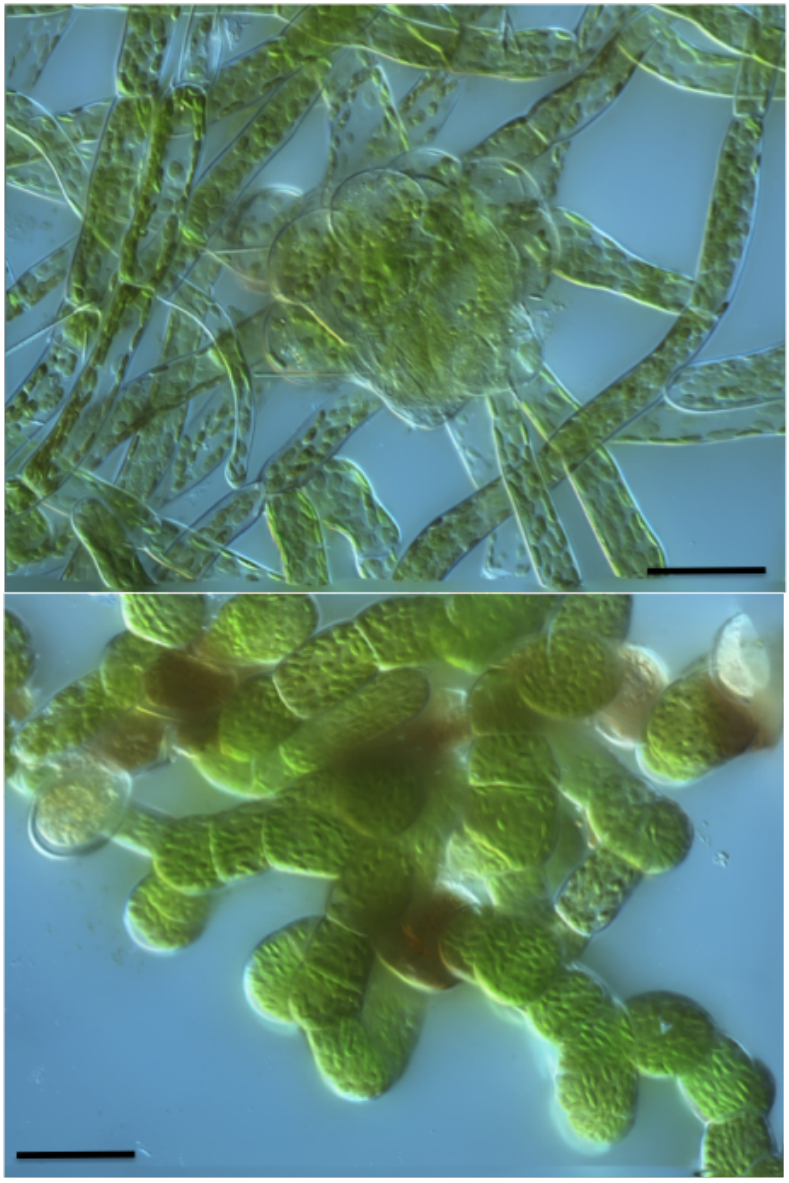
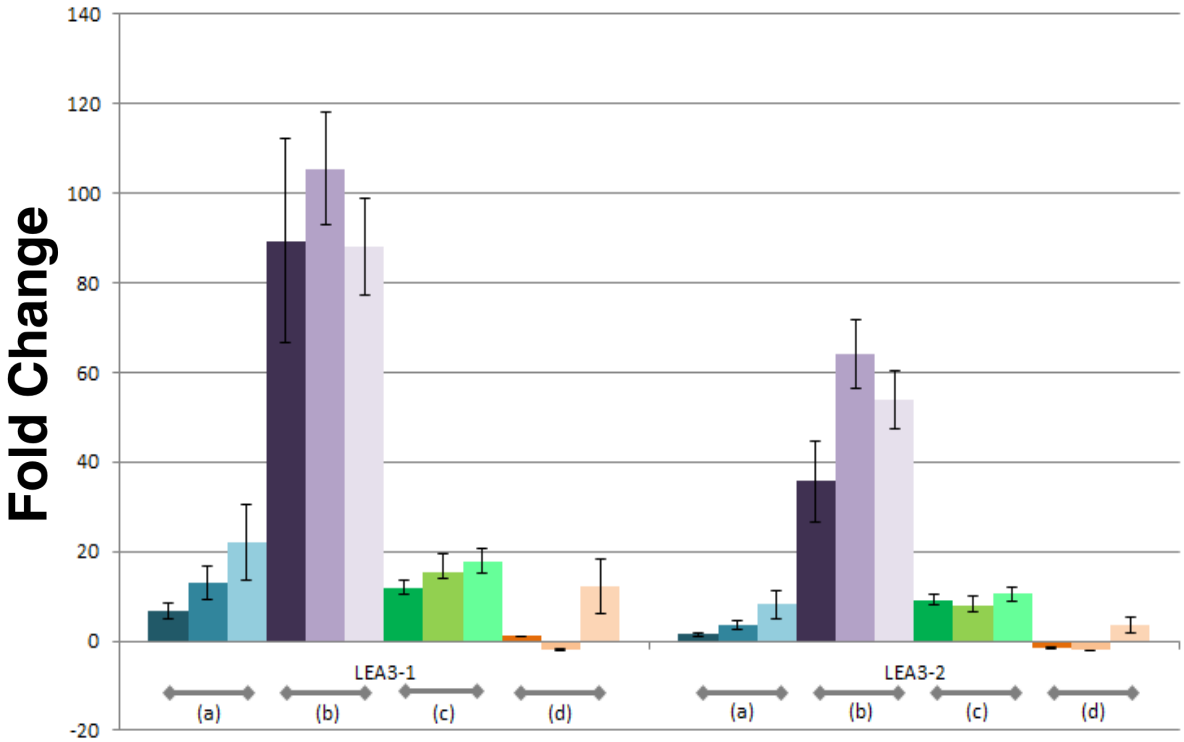


Figure 5.11: ABA treatment phenocopies CCaMK-CYCLOPS gain-of-function lines. Wild-type lines were grown on standard growth media and micrographs taken using differential interference contrast (DIC). (Top) Without ABA treatment, wild-type lines form elongate protonemal cells and interspersed gametophore buds (note centrally located structure). (Bottom) Treatment of wild-type cells with 100 micromolar ABA stimulates brood cell formation and blocks the devlelopment of gametophore buds. Scale bars indicate 50 microns.

Next, we selected two ABA-upregulated marker genes from previous studies on *Physcomitrella* (Shinde *et al*., 2012; Shinde *et al*., 2013) and performed expression analysis using qRT-PCR. These genes encode late embryogenesis abundant (LEA) proteins, which help mitigate dehydration stress by coating other proteins and phospholipids. The selected genes encode group 3 LEA proteins and have been designated *LEA3-1* and *LEA3-2*. The qPCR results showed that ABA marker genes were drastically upregulated in $CYCLOPS_{S107D,S241D}$ lines (Figure 5.12), suggesting that activation of the CCaMK-CYCLOPS signaling module is associated with ABA signaling in moss. Moreover, $CCaMK1_{KD}$ gain-of-function lines arrest growth when treated with ABA at concetrations that are well-tolerated by wild-type cells (Figure 5.13).

Figure 5.12: ABA marker genes are upregulated in CCaMK-CYCLOPS gain-of-function lines. Using qPCR, expression levels of two previously identified ABA-inducible marker genes, labelled LEA3-1 and LEA3-2, were analyzed in transgenic lines. Three independently transformed lines were analyzed. These lines expressed the following genes from the pANIC5A vector: (a) native CYCLOPS, (b) $CYCLOPS_{S107D,S241D}$, (c) CCaMK1, or (d) the calcium indicator GCaMP3 to serve as a negative control. The Y-axis is labelled to indicate fold change in marker gene transcript levels relative to stably expressed reference genes. Positive fold-change values indicate upreglation of the marker gene; negative fold-change values indicated downregulation. The baseline value of zero indicates no change in relative transcript levels. Note the drastic upregulation of ABA marker genes in lines transformed with the dual-phosphomiometic form of CYCLOPS (b, purple).



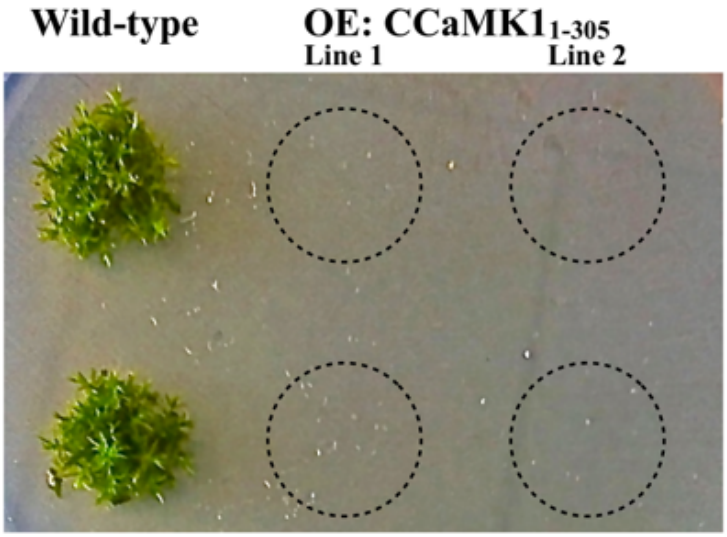
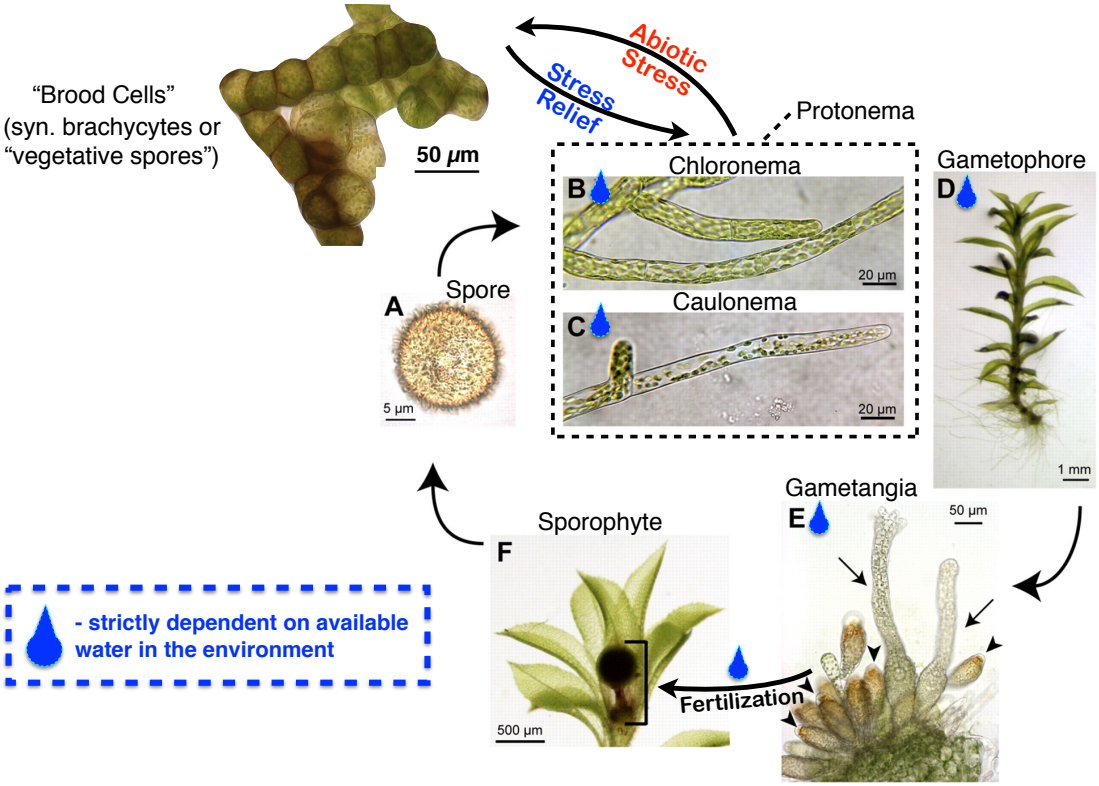


Figure 5.13: CCaMK1$_{KD}$ lines are hypersensitive to treatment with ABA. Equal volumes of freshly blended suspension cultures of wild-type lines or CCaMK1$_{KD}$ lines (amino acids #1-305) were spotted onto standard moss growth media (BCD+NH_4-tartrate) containing 10 micromolar ABA and grown for two months. Whereas wild-type lines were able to establish colonies, CCaMK1$_{KD}$ lines arrested growth.

Although firmly linked to ABA signaling, the molecular genetics of brood cell induction is not well known. One prior study linked a pair of paralogous PP2CA protein phosphatases to brood cell formation, as *pp2ca1 pp2ca2* double knockout lines were shown to form brood cells without the addition of ABA or stress treatments (Komatsu *et al*., 2013). To our knowledge, this is the first study to report genes that act as positive regulators of brood cell formation. Future studies on *ccamk* and *cyclops* loss-of-function mutants in *Physcomitrella* would help to clarify whether the CCaMK-CYCLOPS module is positioned upstream or downstream of ABA signaling in the brood cell induction pathway. From an ecophysiological perspective, brood cell formation is thought to be an important "backup plan" during vulnerable stages of the moss life cycle that enable mosses to survive unfavorable conditions (Figure 5.14). Hence, a signaling module implicated in plant-microbe symbioses regults a key stress response in moss.

The unexpected role of CCaMK-CYCLOPS in stress-associated asexual propagule formation is intriguing given the well-established symbiotic function of CCaMK-CYCLOPS in other plants. It has long been noted that plant-microbe symbiotic associations can be disrupted by abundant nutrient supply (Mosse & Phillips, 1971; Mosse, 1973; Menge *et al*., 1978; Azcón *et al*., 1982; Amijee *et al*., 1989), which could be interpreted as the plant declining the potential symbiotic partner. The mechanism whereby the plant integrates nutrient status and signals from symbiotic partners is unknown. Plants respond to a variety of stresses, including drought, freezing, toxic ion exposure, and nutrient deprivation by accumulating ABA (reviewed by Peleg & Blumwald, 2011). The observed link between CCaMK-CYCLOPS and ABA signaling in moss raises the interesting possibility that ABA may contribute to stress signal integration in the establishment of plant-microbe symbiosis via CCaMK-CYCLOPS. This hypothesis should be further investigated in mycorrhizal-competent plants. Moreover, possible links between brood cell formation and accomodation of microbial symbionts should be investigated in *Physcomitrella* and other mosses.

Figure 5.14: The life cycle of the moss *Physcomitrella patens*. Moss spores germinate to form filamentous cells known as protonema, which are further subdivided into chloronema and caulonema. Protonema form a mat from which leafy shoots known as gametophores arise. The gametophores bear the gametangia in their leaf axils. Fertilization requires that the motile sperm swim from the antheridia to the archegonium in order to reach the egg. Following fertilization, the incipient zygote develops into a stress-resistant spore capsule. From germination until the completion of fertilization, the moss is reliant on available water and nutrients in the environment, therefore it is quite vulnerable. Brood cells, also known as "vegetative spores" serve as stress-resistant propagules formed by protonemal cells exposed to abiotic stress. These brood cells can remain nearly dormant and/or break apart from the colony to relocate. Upon relief from abiotic stress, brood cells germinate to form protonema, and the moss resumes its life cycle.



Sources cited:

Amijee F., Tinker P. B., & Stribley D. P. (1989). The development of endomycorrhizal root systems. *New Phytologist* 111, 435-446.

Ané J. M., Kiss G. B., Riely B. K., Penmetsa R. V., Oldroyd G. E., Ayax C., ... & Cook D. R. (2004). *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. *Science* 303, 1364-1367.

Azcón, R., Gomez Ortega, M., & Barea, J. M. (1982). Comparative effects of foliar or soil applied nitrate on vesicular arbuscular mycorrhizal infection in maize. *New Phytologist* 92, 553-559.

Bécard, G., & Piché, Y. (1990). Physiological factors determining vesicular-arbuscular mycorrhizal formation in host and nonhost Ri T-DNA transformed roots. *Canadian Journal of Botany* 68, 1260-1264.

Bezanilla M., Pan A., & Quatrano R. S. (2003). RNA interference in the moss *Physcomitrella patens*. *Plant Physiology* 133, 470-474.

Brelles-Marino G. Q., & Ané J. M. (2008) Nod factors and the molecular dialogue in the rhizobia–legume interaction. In: *Nitrogen Fixation Research Progress* Ed: Guilherme N. Couto, pp. 1-57 .

Brundrett M.C. (2002). Coevolution of roots and mycorrhizas of land plants. *New Phytologist* 154, 275-304.

den Camp R. O., Streng A., De Mita S., Cao Q., Polone E., Liu W., ... & Geurts R. (2011). LysM-type mycorrhizal receptor recruited for *Rhizobium* symbiosis in nonlegume *Parasponia*. *Science* 331, 909-912.

Capoen, W., Sun, J., Wysham, D., Otegui, M.S., Venkateshwaran, M., Hirsch, S., Miwa, H., Downie, J.A., Morris, R.J., Ané, J.M., & Oldroyd, G.E. (2011) Nuclear membranes control symbiotic calcium signaling of legumes. *Proceedings of the National Academy of Sciences U.S.A.* 108, 14348-14353.

Chabaud M., Genre A., Sieberer B.J., Faccio A., Fournier J., Novero M., Barker D.G., & Bonfante P. (2011) Arbuscular mycorrhizal hyphopodia and germinated spore exudates trigger Ca^{2+} spiking in the legume and nonlegume root epidermis. *New Phytologist* 189, 347-355.

Delaux P.-M., Varala K., Edger P. P., Coruzzi G. M., Pires J. C., & Ané J.-M. (2014). Comparative phylogenomics uncovers the impact of symbiotic associations on host genome evolution. *PLoS Genetics* 10, e1004487.

Endre G., Kereszt A., Kevei Z., Mihacea S., Kaló P., & Kiss G. B. (2002). A receptor kinase gene regulating symbiotic nodule development. *Nature* 417, 962-966.

Frank M. H., & Scanlon M. J. (2015). Transcriptomic evidence for the evolution of shoot meristem function in sporophyte-dominant land plants through concerted selection of ancestral gametophytic and sporophytic genetic programs. *Molecular Biology & Evolution* 32, 355-367.

Gleason C., Chaudhuri S., Yang T., Muñoz A., Poovaiah B. W., & Oldroyd G. E. (2006). Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. *Nature*, *441*(7097), 1149-1152.

Goode J. A., Stead A. D., & Duckett J. G. (1993). Redifferentiation of moss protonemata: an experimental and immunofluorescence study of brood cell formation. *Canadian Journal of Botany* 71, 1510-1519.

Kenrick P., & Crane P.R. (1997). The origin and early evolution of plants on land. *Nature* 389, 33–39.

Kleist T. J., Spencley A. L., & Luan S. (2014). Comparative phylogenomics of the CBL-CIPK calcium-decoding network in the moss *Physcomitrella*, Arabidopsis, and other green lineages. *Frontiers in Plant Science* 5, 187.

Komatsu K., Suzuki N., Kuwamura M., Nishikawa Y., Nakatani M., Ohtawa H., Takezawa D., Seki M., Tanaka M., & Taji T. (2013). Group A PP2Cs evolved in land plants as key regulators of intrinsic desiccation tolerance. *Nature Communications* 4, 2219.

Le Bail A., Scholz S., & Kost B. (2013). Evaluation of reference genes for RT-qPCR analyses of structure-specific and hormone regulated gene expression in *Physcomitrella patens* gametophytes. *PloS One* 8, e70998.

Lévy J., Bres C., Geurts R., Chalhoub B., Kulikova O., Duc G., Journet E.-P., Ané J-.M., Lauber E., & Bisseling T. (2004). A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303, 1361–1364.

Limpens E., Franken C., Smit P., Willemse J., Bisseling T., & Geurts R. (2003) LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302, 630-633.

Maillet F., Poinsot V., André O., Puech-Pagès V., Haouy A., Gueunier M., Cromer L., Giraudet D., Formey D., Niebel A., Martinez E.A., Driguez H., Bécard G. & Dénarié J.

(2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469, 58–63.

Mann D. G. J., LaFayette P. R., Abercrombie L. L., King Z. R., Mazarei M., Halter M. C., Poovaiah C. R., Baxter H., Shen H., & Dixon R. A. (2012). Gateway-compatible vectors for high throughput gene functional analysis in switchgrass (*Panicum virgatum* L.) and other monocot species. *Plant Biotechnology Journal* 10, 226-236.

Menge J. A., Steirle D., Bagyaraj D. J., Johnson E. L. V., & Leonard R. T. (1978). Phosphorus concentrations in plants responsible for inhibition of mycorrhizal infection. *New Phytologist* 80, 575-578.

Mosse B. (1973). Plant growth responses to vesicular-arbuscular mycorrhiza IV: in soil given additional phosphate. *New Phytologist*. 72, 127-136.

Mosse B., & Phillips J. M. (1971). The influence of phosphate and other nutrients on the development of vesicular-arbuscular mycorrhiza in culture. *Journal of General Microbiology* 69, 157-166.

Mylona P., Pawlowski K., & Bisseling T. (1995). Symbiotic nitrogen fixation. *The Plant Cell* 7, 869-885.

Oldroyd G.E.D., & Downie J.A. (2006). Nuclear calcium changes at the core of symbiosis signalling. *Current Opinion in Plant Biology* 9, 351–357.

Oldroyd G.E.D. (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology* 11, 252-263.

Peleg Z., & Blumwald E. (2011). Hormone balance and abiotic stress tolerance in crop plants. *Current Opinion in Plant Biology* 14, 290-295.

Proust H., Hoffmann B., Xie X., Yoneyama K., Schaefer D.G., Yoneyama K., Nogué F., & Rameau, C. (2011) Strigolactones regulate protonema branching and act as a quorum sensing-like signal in the moss *Physcomitrella patens*. *Development* 138, 1531-1539.

Parniske M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology* **6**, 763–775.

Read D. J., Duckett J. G., Francis R., Ligrone R., & Russell A. (2000). Symbiotic fungal associations in 'lower' land plants. *Philosophical Transactions of the Royal Society B: Biological Sciences* 355, 815-831.

Schnepf E., & Reinhard C. (1997). Brachycytes in *Funaria* protonemate: induction by abscisic acid and fine structure. *Journal of Plant Physiology* 151, 166-175.

Shinde S., Nurul Islam M., & Ng C. K. Y. (2012). Dehydration stress induced oscillations in LEA protein transcripts involves abscisic acid in the moss, *Physcomitrella patens*. *New Phytologist* 195, 321-328.

Shinde S., Shinde R., Downey F., & Ng C. K.-Y. (2013). Abiotic stress-induced oscillations in steady-state transcript levels of Group 3 LEA protein genes in the moss, *Physcomitrella patens*. *Plant Signaling & Behavior* 8, e22535.

Singh S., Katzer K., Lambert J., Cerri M., & Parniske M. (2014). CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development. *Cell Host & Microbe* 15, 139-152.

Svistoonoff S., Benabdoun F. M., Nambiar-Veetil M., Imanishi L., Vaissayre V., Cesari S., ... & Gherbi H. (2013). The independent acquisition of plant root nitrogen-fixing symbiosis in fabids recruited the same genetic pathway for nodule organogenesis. *PloS One* 8, e64515.

Tirichine L., Imaizumi-Anraku H., Yoshida S., Murakami Y., Madsen L. H., Miwa H., Nakagawa T., Sandal N., Albrektsen A. S., & Kawaguchi M. (2006). Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* 441, 1153-1156.

Veiga R. S., Faccio, A., Genre, A., Pieterse, C. M., Bonfante, P., & Heijden, M. G. (2013). Arbuscular mycorrhizal fungi reduce growth and infect roots of the non-host plant *Arabidopsis thaliana*. *Plant, Cell & Environment* 36, 1926-1937.

Wang B., & Qiu Y. L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* 16, 299-363.

Wang B, Yeun LH, Xue JY, Liu Y, Ané JM, & Qiu YL (2010). Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. *New Phytologist* 186, 514–525.

Yano K., Yoshida S., Müller J., Singh S., Banba M., Vickers K., ... & Parniske M. (2008). CYCLOPS, a mediator of symbiotic intracellular accommodation. *Proceedings of the National Academy of Sciences U.S.A.* 105, 20540-20545.

Chapter 6: Identification of a new family of calcium-permeable ion channels found in plants, fungi, and metazoans.

The following chapter (excluding the preface) was published as a peer-reviewed research article in the journal *Cell Research* and is reproduced here with permission and contains minor editorial changes.

Hou C.*, Tian W.*, Kleist T.J.*, He K., Garcia V., Bai F., Hao Y., Luan S., & Li L. (2014). "DUF221 proteins are a family of osmosensitive calcium-permeable cation channels conserved across eukaryotes." *Cell Research* 24, 632-635.

* These authors contributed equally to this work

Contributions.

T.J. Kleist helped to interpret the results of electrophysiological experiments, performed all described computational biology experiments, and served as primary writer of the manuscript. C. Hou and W. Tian helped to design experiments and performed electrophysiological and calcium imaging experiments with technical assistance from F. Bai and Y. Hao. K. He and V. Garcia provided technical assistance and helped to interpret experimental results. S. Luan and L. Li guided experimental design, advised with interpreting experimental results, and helped write the manuscript.

Preface:

Calcium signals can be evoked in plant cells by myriad stimuli, ranging from abiotic parameters such as temperature to biotic factors emitted by plants or other organisms. Previous chapters have largely focused on efforts to characterize protein kinases that putatively act in calcium-decoding networks. The plant biology community has made considerable progress on the characterization of the plant calcium signal-decoding network; however, the mechanisms underlying signal coding remain obscure. Calcium signals can be monitored *in vivo* using electrophysiological or optical methods. Calcium signals can be visualized using genetically encoded calcium indicators or any of a number of fluorescent dyes that can be loaded into living cells. In the following chapter, the latter principle was leveraged to screen genes from Arabidopsis predicted to encode integral membrane proteins in a heterologous system, Chinese hamster ovary (CHO) cells. After observing that a protein containing domain of unknown function 221 (DUF221) appeared to show osmosensitive cation channel activity in CHO cells or *Xenopus* oocytes, computional analyses showed that DUF221-containing proteins constitute a previously unnoticed family of ion channels with permeability to calcium (Ca^{2+}) that are conserved across eukaryotes. Loss-of-function mutant analyses were pursued extensively in Arabidopsis and *Saccharomyces cerevisiae*, which contains four DUF221-containing proteins; however these failed to yield any discernible phenotype. The function(s) of DUF221 proteins, which we named calcium-permeable stress induced cation channels (CSCs) therefore remains obscure, however they are

interesting candidates for a role in calcium-mediated osmosensing. The phylogenomic distribution of CSCs is provided in the following chapter along with evidence for the ion channel activities of CSCs from Arabidopsis, *S. cerevisiae*, and human.

Introduction:

The flow of ions through channels in cell membranes, particularly the entry of calcium ions into the cytosol, serves as a cue for environmental responses in eukaryotes. In animals, calcium-permeable transient receptor potential (TRP) channels act as sensors for temperature, osmotic potential, and other environmental conditions (Venkatachalam *et al*., 2007). Although plants also respond to myriad environmental perturbations with cytosolic calcium fluxes (Knight *et al*., 1991; Knight *et al*., 1997), stress- gated calcium-permeable channels like TRPs in animals are not found in land plants. Several families of proteins, including glutamate receptor-like (GLR) proteins, cyclic nucleotide-gated channels (CNGCs), and annexins, have been linked to calcium fluxes in plant cells (Swarbreck *et al*., 2013). However, none of them has been shown to respond directly to stress signals.

Because cytosolic calcium elevation is one of the earliest responses of plant cells to stress treatments (Knight *et al*., 1991; Knight *et al*., 1997) and calcium-binding proteins are required for several environmental stress responses (Luan *et al*., 2002), stress-activated calcium channels are candidates that may link stress stimuli to calcium-dependent downstream responses. We thus seek to isolate plant genes that encode possible calcium channels that are gated by stress signals, such as osmotic stress.

Materials & Methods:

Oocyte Electrophysiology.

The full-length cDNAs of AtCSC1, HsCSC1 and ScCSC1 were subcloned into vector pGEMHE (Liu & Luan, 2001). cRNA was synthesized from 1 µg of linearized plasmid DNA template using an mMessage mMachine in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Defolliculated *Xenopus laevis* oocytes were injected with 23 ng and incubated in ND96 at 18 °C for 4 days prior to electrophysiological assays. Oocytes were voltage-clamped at a holding potential of –60 mV using a TEV 200 amplifier (Dagan, Minneapolis, MN) and monitored by computer through a Digidata 1440A/D converter and pCLAMP 10.2 software (Axon Instruments, Foster City, CA). The pipette solution contained 3 M KCl. The oocytes were superfused with a standard ND96 solution containing (in mM) 96 NaCl, 2.0 KCl, 1.0 MgCl2, 1.8 $CaCl_2$ and 10 HEPES, pH 7.5, at room temperature. For cation substitution experiments, 96 mM NaCl was replaced with 96 mM KCl or $CaCl_2$. In calcium-free bath solution, 1.8 mM $CaCl_2$ was replaced with 0.5 mM EGTA. Hyperosmotic stimuli were applied by perfusion with bath solution containing the indicated concentrations of mannitol.

Cell Lines and Ca^{2+}-imaging Experiments.

The complete cDNAs of AtCSC1, HsCSC1 and ScCSC1 were subcloned into eukaryotic expression vector pcDNA3.0 and transfected into CHO-K1 cells using Fugene 6 (Roche Applied Science, Indianapolis, IN). The transfected cells were cultured in DMEM liquid media containing 10% FBS (GIBCO, Grand Island, NY) and enriched with 10% CO_2, with the addition of 1 mg/ml pen-strep (Sigma-Aldrich, St. Louis, MO) and 800 µg/ml G418 for selection. Stable clones that expressed the proteins were confirmed by western blot.

Cells were plated on glass-bottom dishes with DMEM liquid culture medium containing 10% fetal bovine serum (GIBCO, Grand Island, NY) and and enriched with 10% CO_2, with the addition of 1 mg/ml pen-strep (Sigma-Aldrich, St. Louis, MO). Cells were incubated for 48 hours at 37°C prior to experiments. The Ca^{2+}-imaging experiments were performed following procedures similar to Liedtke et al. (2000), with minor modifications as described by Liu & Luan (2001). After removal of culture medium, cells were loaded with Fura-2 by incubation at room temperature with 10 µM Fura-2 acetoxymethyl ester dissolved in isosmotic saline solution, which contains (in mM) 130 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 D-glucose, 10 HEPES, pH 7.4, and 0.02% pleuronic acid (Molecular Probes, Eugene, OR). Following loading with Fura-2, cells were washed four times with saline solution and allowed to recover in 1 mL saline solution for 15 minutes at 37°C. After recovery, cells were imaged at 2-second intervals for 480 seconds in total to monitor cytosolic calcium status. At 120 seconds, hyperosmotic shock was applied by addition of 1 mL saline solution containing 600 mM mannitol (300 mM final mannitol concentration). At 360 seconds, the medium was restored to near-isosmotic conditions by addition of 2 mL H_20. The Ca^{2+} concentration of

all solutions was held constant at 2 mM. Images of Fura-2 loaded cells with the excitation wavelength alternating between 340 nm and 380 nm were captured with a CCD camera. After subtraction of background fluorescence, the ratio of fluorescence intensity at the two wavelengths was calculated. The ratio of 340/380 nm fluorescence intensity in groups of 10-20 individual cells with the strongest responses were analyzed using MetaFluor (Universal Imaging Corporation). All graphs are the averaged responses from groups of individual cells from representative single experiments.

Mammalian Cell Electrophysiology.

CHO cells were plated onto poly-D-lysine-coated coverslips for recording purposes, and recordings were performed 24 h later. Experiments were carried out at room temperature using whole-cell voltage clamp technique, with an Axopatch 2B amplifier filtered at 5 kHz and pClamp suite of software (Axon Instruments, Foster City, CA). Series-resistant compensation was 80% for all experiments, using 2–5 MΩ fire-polished pipettes. Pipette solution contained, in mM, 140 CsCl, 1 EGTA, 10 HEPES, 2 MgATP, pH 7.4 (adjusted with CsOH). The isosmotic bath solution contained (mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4 (adjusted with NaOH). During whole-cell recording, cells were maintained in isosmotic bath solution. Osmotic shock was accomplished by perfusion with hypertonic solution (the bath solution plus 500 mM mannitol).

Bioinformatics.

Using the amino acid sequence of AtCSC1 (UniProt: Q5XEZ5) as a query, homologous sequences were identified in UniProt and NCBI protein databases using the BLASTp algorithm. Sequences were aligned to create a multiple sequence alignment (MSA) using MAFFT v1.3 3 with the BLOSUM62 scoring matrix and E-INS-i algorithm. The resulting MSA was edited manually and ambiguously aligned regions were masked. The edited and masked MSA was used to generate a maximum likelihood (ML) phylogenetic tree using PhyML v2.2.0 (Guindon *et al*., 2010) with the following parameters: WAG+G model, best of NNI + SPR search, and chi-squared-like approximate likelihood ratio test (aLRT) clade support statistics (Anisimova & Gascuel, 2006). Membrane topology for DUF221-containing proteins was predicted using MetaTM (Klammer *et al*., 2009). Bioinformatic analyses were conducted using Geneious v6.1.4 created by Biomatters (76 Anzac Ave, Auckland, 1001, New Zealand).

Results & Discussion:

Using heterologous expression of *Arabidopsis* genes in Chinese Hamster Ovary (CHO) cells loaded with the calcium-responsive dye Fura-2, we screened uncharacterized integral membrane proteins for osmosensitive calcium conductance. These screens led to the identification of the *Arabidopsis* gene *At4G22120*, which encodes an integral membrane protein with eight or more predicted transmembrane helices. Ratiometric monitoring of Fura-2 fluorescence indicated that hyperosmotic shock treatment applied by 300 mM mannitol treatment induced calcium elevation in CHO cells expressing At4G22120 (UniProt: Q5XEZ5), whereas cells containing the empty vector control lacked discernible calcium elevation (Figure 6.1A). The observed calcium elevation occurred rapidly, peaking several seconds after hyperosmotic shock, and the calcium level sharply declined within 1 minute of osmotic shock (Figure 6.1B).

Because *At4G22120* encodes a protein with multiple predicted transmembrane helices, we tested the possibility that it may form an influx cation channel permeable to calcium. We expressed the protein by injecting cRNA of this gene into *Xenopus* oocytes and performed whole-cell two-electrode voltage clamp (TEVC) analysis. Oocytes expressing the cRNA of the gene were placed in ND96 bath solution, and hyperosmotic shock was applied by perfusion with ND96 solution containing 500 mM mannitol. Hyperosmotic shock induced an inward current indicative of channel activation, and the

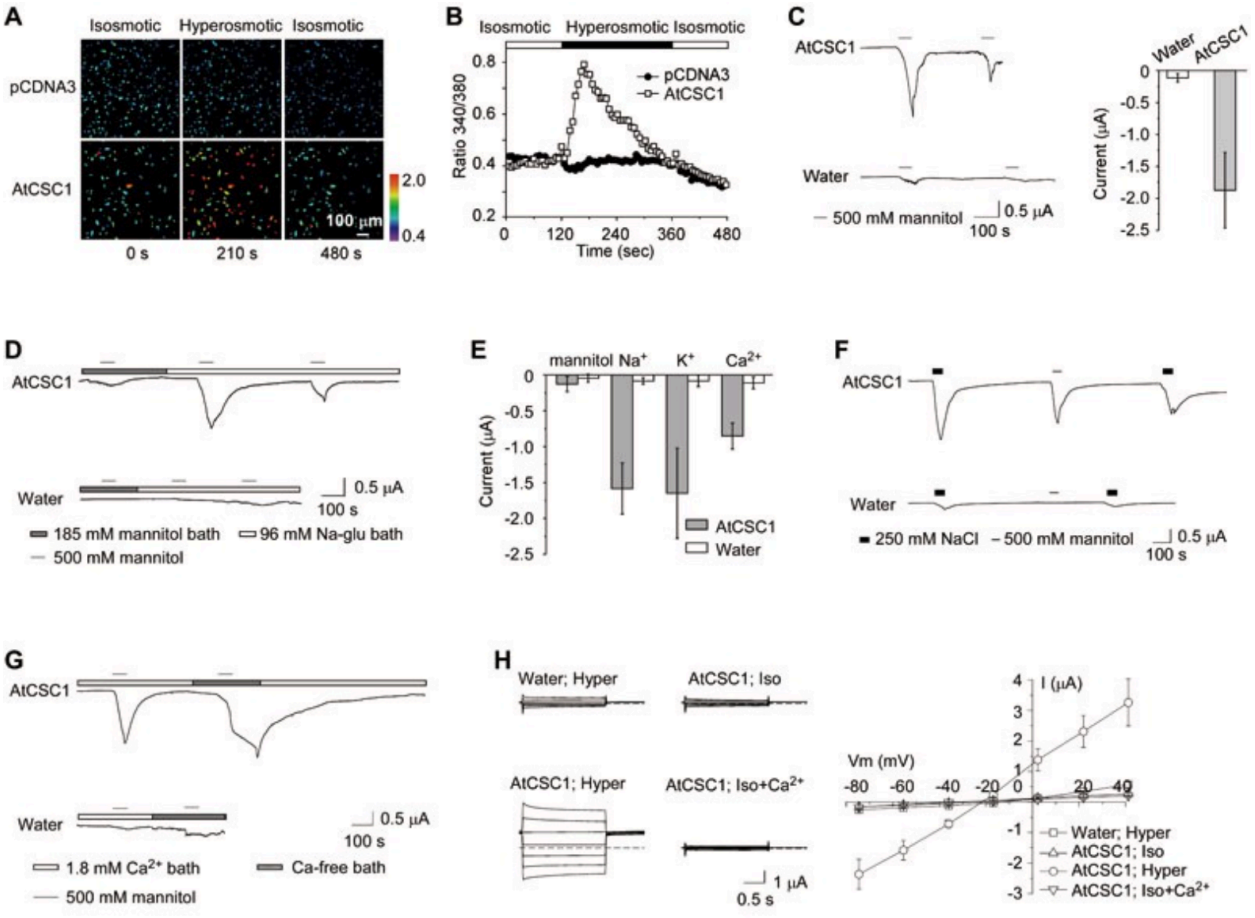


Figure 6.1: Characterization of CSCs as a family of cation channels by electophysiological analyses in heterologous systems and bioinformatic approaches. (A) Representative images taken from CHO cells transfected with empty pCDNA3 vector or the same vector harboring AtCSC1. These images show that AtCSC1-expressing CHO cells respond to hyperosmotic stress by cytosolic $[Ca^{2+}]_i$ elevation not seen in control cells. $[Ca^{2+}]_i$ changes are indicated by Fura-2 emission ratios ($F_{340\ nm}/F_{380\ nm}$) scaled by a pseudo-color bar in relative units. Hyperosmotic stress was applied by including 300 mM mannitol in isoosmotic saline solution. **(B)** Time course of intracellular calcium $[Ca^{2+}]_i$ changes in AtCSC1-expressing CHO cells. Open and filled bars show the isoosmotic and hyperosmotic conditions, respectively. The average response of 10-20 cells from a representative experiment is presented. **(C)** (left) Typical whole-cell current traces recorded from AtCSC1-expressing *Xenopus* laevis oocytes (AtCSC1) and the water-injected control oocyte (water) with periodic perfusion of saline solution containing 500 mM D-mannitol. (right) Current amplitudes evoked by the first application of hypersosmotic stress (n = 5). **(D)** Typical whole-cell current traces recorded from the AtCSC1-expressing oocytes perfused with bath solution containing 185 mM D-mannitol or 96 mM Na-gluconate (Na-glu) plus 500 mM D-mannitol. **(E)** Current amplitudes produced by 96 mM Na^+ or K^+ or 80 mM Ca^{2+}. **(F)** AtCSC1-produced inward current was activated by equal osmolality of NaCl or mannitol. **(G)** AtCSC1 failed to inactivate under calcium-free extracellular conditions, implicating calcium-dependent channel closure. **(H)** (left) Typical whole-cell current traces recorded from the water-injected control and AtCSC1-expressing oocytes perfused with calcium-free isoosmotic buffer (Iso), calcium-free hyperosmotic buffer (Hyper) or isoosmotic buffer with 1.8 mM Ca^{2+} (Iso + Ca^{2+}). (right) Current/voltage (I/V) relationship of the same recordings. The current values were extracted at the end of 2 s voltage pulses (n = 5). The bars represent means ± SD. The membrane potential was held at 0 mV and stepped in 20 mV increments from –80 to +40 mV.

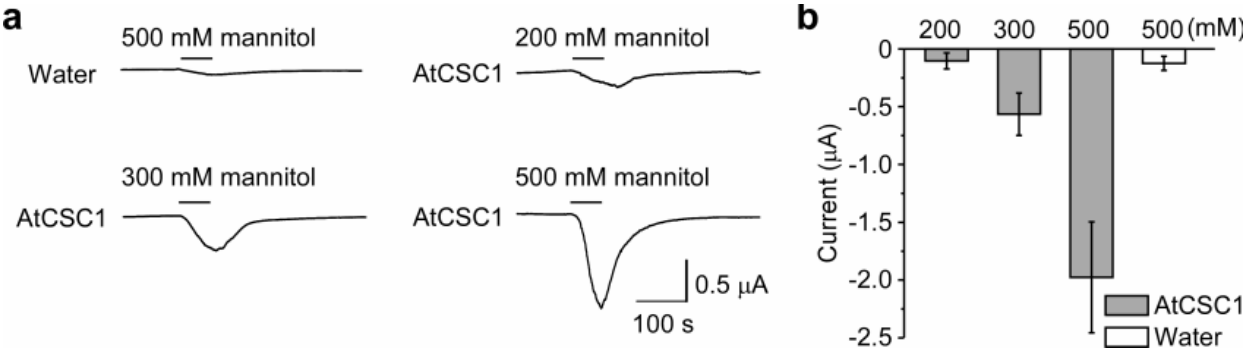
observed current disappeared upon removal of osmotic shock, implicating channel closure (Figure 6.1C). The second osmotic shock induced a smaller inward current, suggesting response dampening upon repeated stimulation. These results indicate that the At4G22120 protein is an ion channel that can be activated by hyperosmotic shock. We therefore named this protein AtCSC1 as *Arabidopsis* Calcium-permeable Stress-gated cation Channel 1.

As expected of a channel, the current was barely detectable when equal molarity of mannitol (185 mM) was used to replace the major charge carrier NaCl in the ND96 solution, even after the same hyperosmotic shock (Figure 6.1D). Replacement of 185 mM mannitol with 96 mM Na^+-gluconate restored an inward current in response to the hyperosmotic treatment (Figure 6.1D), suggesting that the channel specifically conducts cations (i.e., Na^+) rather than the much larger gluconate anions. Further analyses revealed that AtCSC1 is permeable to various cations, including Ca^{2+}, K^+, and Na^+ (Figure 6.1E).

To further investigate AtCSC1 activation, we applied hyperosmotic stress to AtCSC1-expressing oocytes using various concentrations of mannitol or NaCl. Channel conductance showed dependence on D-mannitol concentration in the extracellular buffer, with 300 mM or higher concentrations sufficient for channel activation (Figure 6.2). Hyperosmotic shock could be applied with 250 mM NaCl substituted for 500 mM mannitol, and it similarly activated AtCSC1 (Figure 6.1F). The currents induced by mannitol or NaCl treatment abated quickly after removal of hyperosmotic shock. This observation piqued our interest in closure of the AtCSC1 ion channel, therefore we tested the hyperosmotic shock responses of oocytes expressing AtCSC1 with 500 mM mannitol under calcium-free conditions. The channel activated in response to hyperosmotic shock but failed to inactivate after removal of osmotic shock until perfusion was resumed with ND96 solution containing 1.8 mM Ca^{2+} (Figure 6.1G), indicating that inactivation or closure of the AtCSC1 channel is calcium-dependent. The currents conducted by AtCSC1 occurred instantaneously upon application of hyperosmotic shock and were time-independent (i.e., activation was not gradual). Stepwise voltage analysis indicated that the channel is non-rectifying (Figure 6.1H).

A previous proteomics study indicates that AtCSC1 localizes to the plasma membrane (Nuhse *et al.*, 2003), which is consistent with its apparent localization in our CHO and oocyte experiments. According to hydrophobicity plots and the MetaTM webserver (http://metatm.sbc.su.se), which predicts transmembrane protein topology using a consensus approach of popular transmembrane helix prediction algorithms, AtCSC1 contains two clusters of transmembrane (TM) helices (Figure 6.3A). The N-terminal region contains three predicted TM helices, however we postulate that the first predicted helix may act as a cleavable signal peptide. The C-terminal region comprising domain of unknown function 221 (DUF221) contains six predicted TM helices but lacks discernible sequence similarity to other characterized cation channels. Overall, the AtCSC1 protein likely contains eight TM helices in total and may constitute the pore-forming entity of an ion channel. The two clusters of TM helices appear to be linked by a roughly 200 amino acid cytosolic loop.

Figure 6.2: Concentration-dependent Activation of AtCSC1 by D- mannitol. (a) Typical whole-cell current traces recorded from the AtCSC1-expressing oocytes treated with different concentrations of D-mannitol. **(b)** The current amplitudes evoked by 200 mM, 300 mM and 500 mM D-mannitol (n=5). The bars represent means ±s.d.



Homologs of AtCSC1 with conserved membrane topology were identified among nearly all studied eukaryotes, including baker's yeast, fruit flies, and humans. Phylogenetic analyses revealed that land plants contain 4 ancient clades of *AtCSC1* homologs and that *Arabidopsis* contains at least 15 CSCs (Figures 6.3B, 6.6 – 6.8). We identified four CSC homologs in the model yeast *Saccharomyces cerevisiae* and cloned one of these homologs, *ScYLR241W,* for heterologous expression and electrophysiological characterization. Like AtCSC1, ScYLR241W showed osmotically gated calcium conductance when heterologously expressed in CHO cells and similar electrophysiological properties to AtCSC1 in oocytes (Figure 6.4), therefore we named this protein ScCSC1. Three AtCSC1 orthologs were identified among all sequenced vertebrates. We characterized one homolog from human, HsTM63C (UniProt: Q9P1W3), in CHO cells and *Xenopus* oocytes, as described for AtCSC1 and ScYLR241W. HsTM63C showed similar gating properties and conductance to AtCSC1 and ScYLR241W, therefore we designated this gene *HsCSC1* (Figure 6.4). These data for ScCSC1 and HsCSC1 provide further support for our hypothesis that CSC homologs constitute a previously unidentified family of osmosensitive, calcium-permeable cation channels in eukaryotes.

Published expression data indicate that several *Arabidopsis* CSCs are transcriptionally upregulated in response to various abiotic stresses and biotic stresses, which both entail mechanical perturbation. Indeed, some CSC proteins were previously named Early Response to Dehydration (ERD) 4 proteins based on their expression profile (Kiyosue *et al*., 1994). According to Pfam, DUF221 belongs to the anoctamin-like clan and is homologous to domains in anoctamin/TMEM16 channels, which are calcium-activated chloride channel (CaCC) components (Schroeder *et al*., 2008), and transmembrane channel-like (TMC) proteins. TMCs are cation channel components, and certain TMCs are required for salt chemosensation in *C. elegans* (Chatzigeoriou *et al*., 2013) and hearing in mammals (Pan *et al*., 2013). Our data establish that CSCs are a family of cation channels that are permeable to calcium and gated by physical signals such as hyperosmotic stress. These properties are reminiscent of TRPs in animals and make CSCs intriguing candidates for involvement in osmo- or mechano-sensitive calcium signaling processes in plants. Given their conservation in distantly related eukaryotes including fungi and animals, this study opens new avenues for research towards understanding the function of this novel family of ion channels not only in plants but also in other eukaryotes.

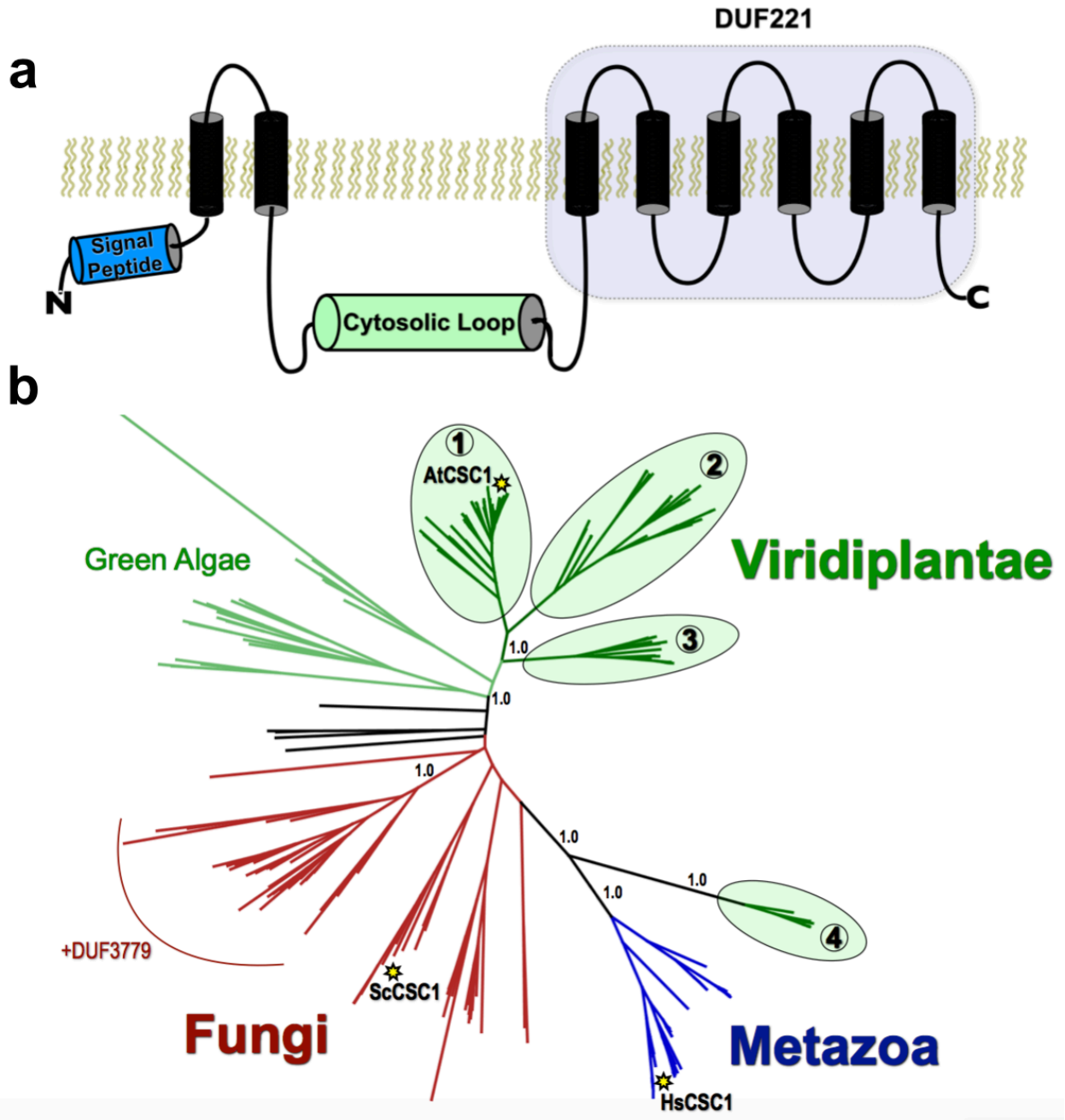


Figure 6.3: (a) Predicted transmembrane topology of AtCSC1 and CSC homologs; the first predicted transmembrane helix is hypothetically a cleavable signal peptide. **(b)** ML phylogenetic tree of DUF221-containing proteins created using PhyML v.2.2.0. Yellow stars mark the positions of AtCSC1, ScCSC1, and HsCSC1. Land plants contain four clades of CSCs, including one clade that clusters with metazoan homologs. One fungal clade contains an additional DUF3779, which is located at the C-terminus of these homologs. Support values (aLRT) are labeled for select clades.

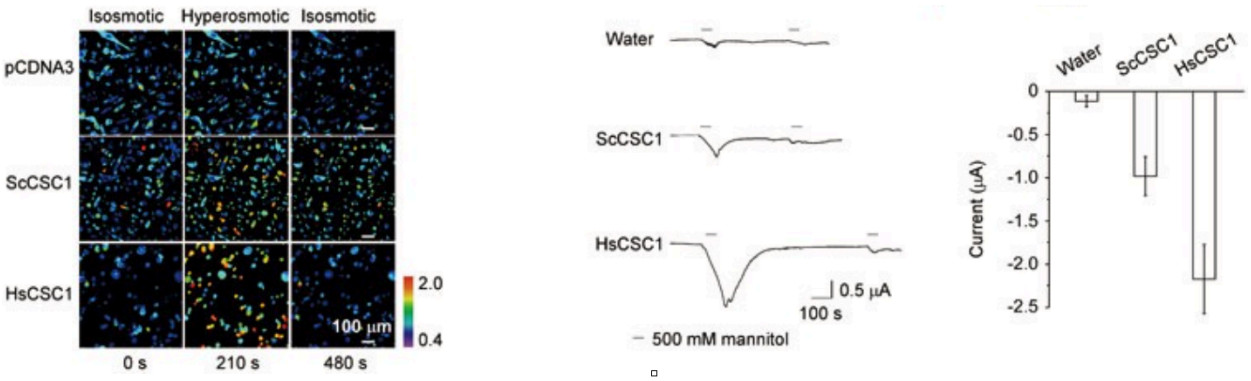
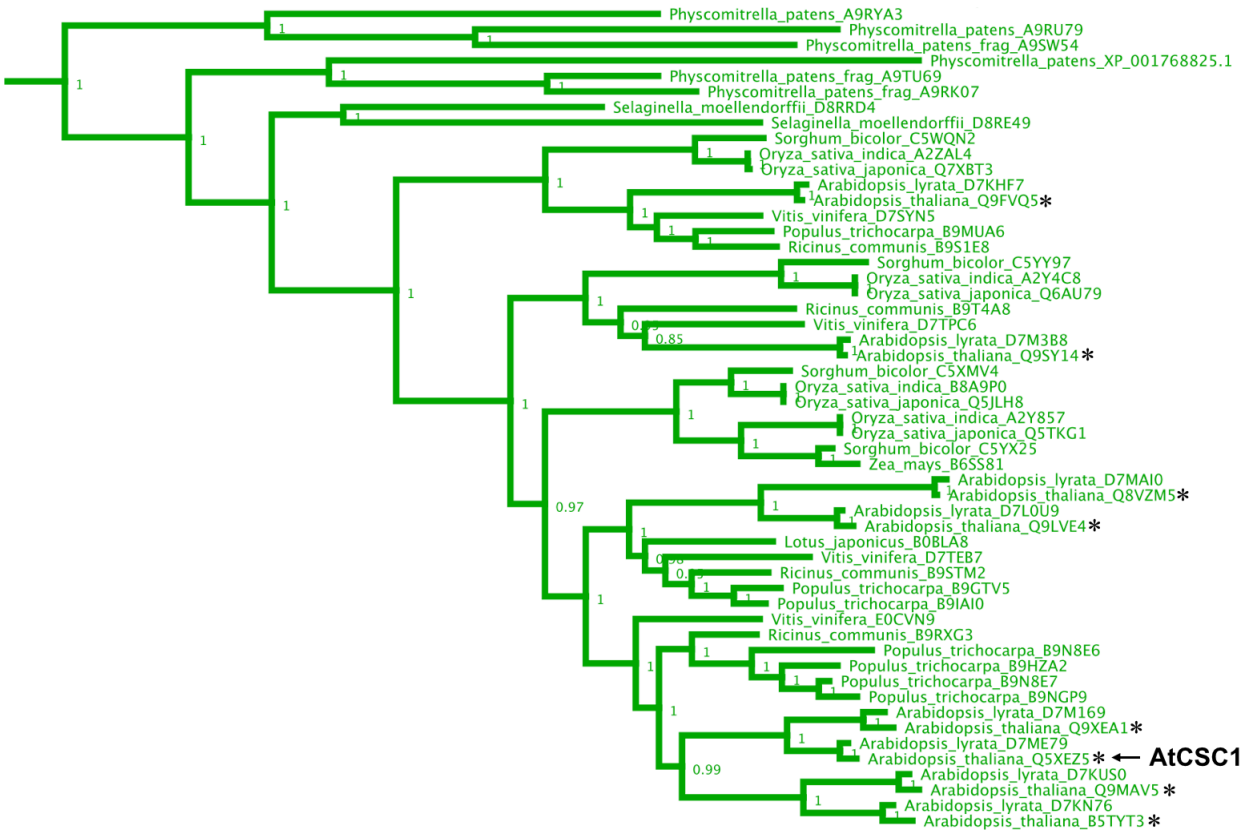


Figure 6.4: (Left) Representative images taken from Fura-2-loaded CHO cells expressing ScCSC1 or HsCSC1 after they were treated with isoosmotic saline solution or hyperosmotic solution containing 300 mM mannitol. These data indicate that yeast and human CSC proteins are also osmosensitive, calcium-permeable cation channels. (Middle) Typical whole-cell current traces recorded from water-injected or ScCSC1- or HsCSC1-expressing oocytes with periodic application of 500 mM D-mannitol. (Right) The current amplitudes evoked by the first application of 500 mM D-mannitol (n = 5). The bars represent means ± SD. The holding potential was –60 mV.

Figure 6.5: Close-up of clade 1 shown in Figure 6.3B. This clade contains 8 of the 15 identified Arabidopsis CSC1 homologs (marked with asterisks). The large number of homologs contained in this clade suggests there may be functional redundancy among these members.



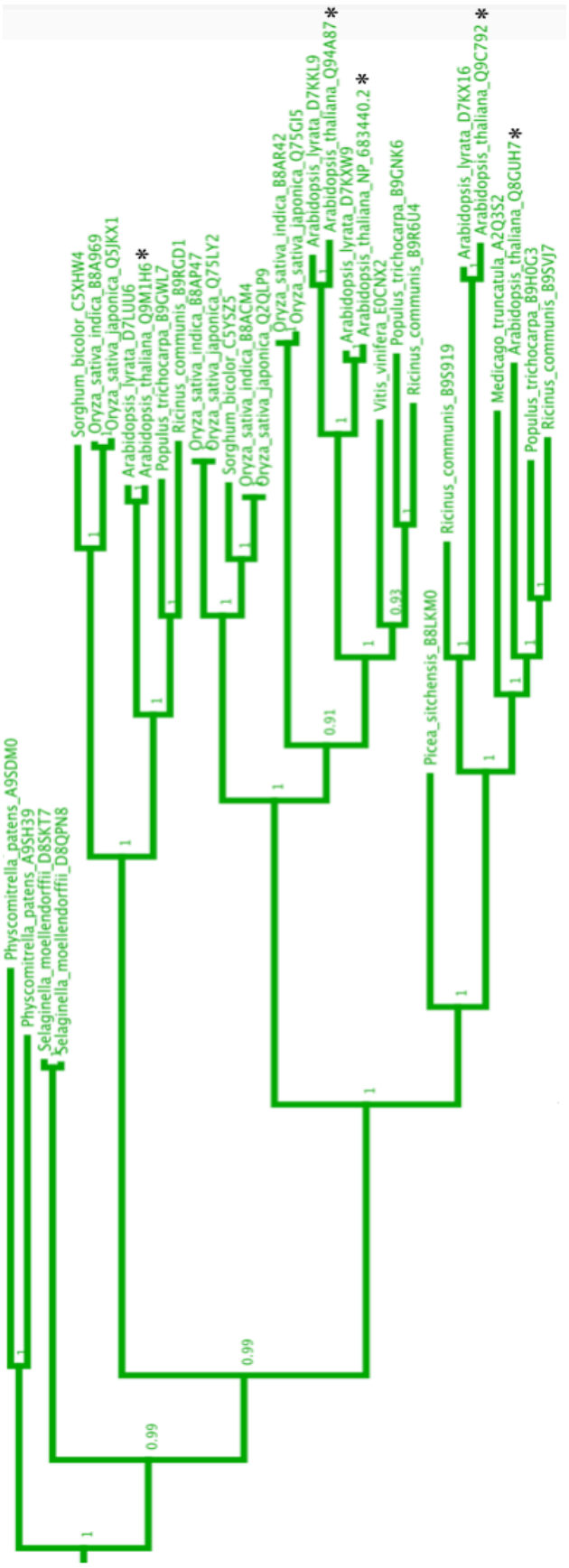


Figure 6.6: Close-up of clade 2 from Figure 6.3B. This clade contains 5 of the 15 identified Arabidopsis CSC1 homologs (marked with asterisks).

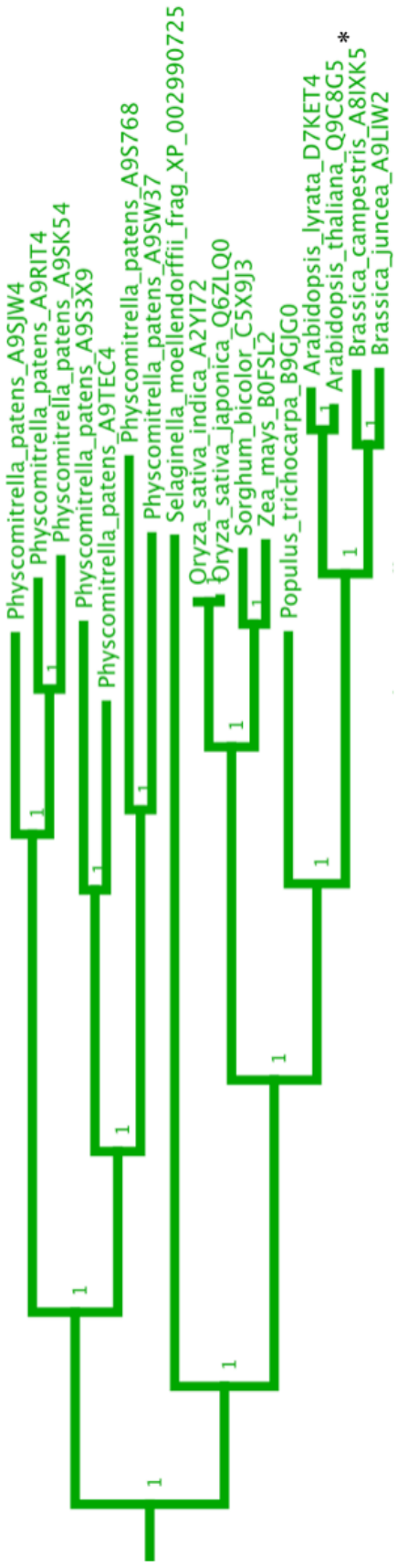


Figure 6.7: Close-up of clade 3 from Figure 6.3B. This clade contains only a single representative from Arabidopsis (marked with an asterisk).

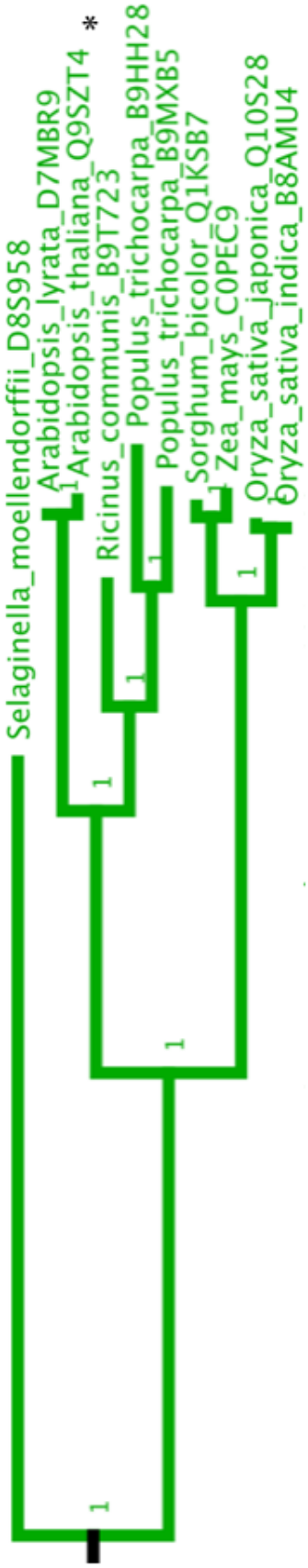


Figure 6.8: Close-up of clade 4 from Figure 6.3B. This clade a single representative from Arabidopsis (marked with an asterisk) and, unlike clades 1 – 3, does not contain any representatives from the moss *Physcomitrella*. Unlike other plant CSC homologs, members of this clade are predicted to be intronless.

Sources Cited:

Anisimova M., & Gascuel O. (2006) Approximate Likelihood-Ratio Test for Branches: A Fast, Accurate, and Powerful Alternative. *Systematic Biology* 55, 539–552.

Chatzigeorgiou M., Bang S., Hwang S. W., & Schafer W. R. (2013). *tmc-1* encodes a sodium-sensitive channel required for salt chemosensation in *C. elegans*. *Nature* 494, 95-99.

Guindon S., Dufayard J. F., Lefort V., Anisimova M., Hordijk W., & Gascuel O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* 59, 307-321.

Katoh K., Misawa K., Kuma K. I., & Miyata T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* 30, 3059–3066.

Kiyosue T., Yamaguchi-Shinozaki K., & Shinozaki K. (1994). Cloning of cDNAs for genes that are early-responsive to dehydration stress (ERDs) in *Arabidopsis thaliana* L.: identification of three ERDs as HSP cognate genes. *Plant Molecular Biology* 25, 791-798.

Klammer M., Messina D. N., Schmitt T. & Sonnhammer E. L. L. (2009) MetaTM - a consensus method for transmembrane protein topology prediction. *BMC Bioinformatics* 10, 314.

Knight H, Trewavas AJ, & Knight MR. (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *The Plant Journal* 12, 1067–1068.

Knight MR, Campbell AK, Smith SM, & Trewavas, A.J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352, 524–526.

Liedtke W., Choe Y., Martí-Renom M. A., Bell A. M., Denis C. S., Hudspeth A. J., ... & Heller S. (2000). Vanilloid receptor–related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* 103, 525-535.

Liu K., & Luan S. (2001) Internal aluminum block of plant inward K(+) channels. *The Plant Cell* 13, 1453–1465.

Luan S., Kudla J., Rodriguez-Concepcion M., Yalovsky S., & Gruissem W. (2002). Calmodulins and calcineurin B-like proteins calcium sensors for specific signal response coupling in plants. *The Plant Cell* 14, S389-S400.

Nühse T. S., Stensballe A., Jensen O. N., & Peck S. C. (2003). Large-scale analysis of *in vivo* phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. *Molecular & Cellular Proteomics* 2, 1234-1243.

Schroeder B. C., Cheng T., Jan Y. N., & Jan L. Y. (2008). Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. *Cell* 134, 1019-1029.

Pan B., Géléoc G. S., Asai Y., Horwitz G. C., Kurima K., Ishikawa K., ... & Holt J. R. (2013). TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. *Neuron* 79, 504-515.

Swarbreck SM, Colaço R, & Davies, JM. (2013) Plant calcium-permeable channels. *Plant Physiology* 163, 514-522.

Venkatachalam K., & Montell C. (2007) TRP channels. *Annual Review of Biochemistry* 76, 387-417.

Chapter 7: Concluding remarks.

Calcium signals are a pervasive mechanism for environmental perception in eukaryotes. Plants respond to staggeringly diverse signals with nucleocytoplasmic calcium signals, suggesting that calcium signals act as a code that keeps plants appraised of their environmental status. Calcium-binding motifs that enable calcium signal decoding, such as EF-hands, can be readily identified by bioinformatic methods. This principle was leveraged here to computationally analyze calcium signaling components in *Physcomitrella patens*, currently the only moss species with a published genome sequence. Clearly, advances in genome and transcriptome sequencing technology have provided powerful tools to analyze gene families in diverse plant lineages. Improved coverage for charophyte green algae and early-diverging land plants would greatly aid future phylogenomic studies. Nonetheless, moving forward, many questions linger, particularly upstream of calcium signaling events, that cannot be addressed by 'omics approaches. Calcium-permeable channels may respond directly to environmental stimuli and thereby directly function as sensors, or they may be gated by upstream signals, or they may be regulated by both mechanisms. Despite tremendous effort, very few candidate sensor-channel families have been identified in plants, as discussed in greater detail in Chapters 2 and 6. Functional redundancy — either within or among gene families — may underly some of the difficulties of investigating the topic in plants.

Advancements in optical methods might partly address difficulties investigating channels responsible for identifying ion channels responsible for calcium signal generation in plants. Plant cell walls pose challenges for the use of dyes, however there have been considerable advances recently with the use of genetically encoded calcium indicators (GECIs) in plants (reviewed by Swanson *et al*., 2011; Shaw & Ehrhardt, 2013). Coupled with advances in microscope technology, this vein of approaches is promising. It would be interesting to use our knowledge of calcium-regulated protein kinases, for example CBL-CIPK complexes or CCaMKs, in order to design GECIs that are targeted to subcellular locations that correspond to the native complexes, in order to allow monitoring of calcium signals that are decoded by specific complexes. CBLs might be particularly suited to test this idea by using the distinctive N-terminal localization signals to target GECIs to membrane locations. Close inspection of calcium signals at precise subcellular locations may enable researchers to identify previously unnoticed phenotypes in various loss-of-function lines and could complement growth assays, which have so far yielded few clues about calcium signal-coding systems in plants.

Molecular genetic work in native plant systems under investigation clearly remains the gold standard. There is an urgent need to simplify and expedite transformation technologies in diverse plants to enable gene functional investigation. The optimization of transformation protocols for *Physcomitrella* transformation described in Chapter 4 enabled functional investigation of CCaMK and its target, CYCLOPS, and revealed its unexpected role in stress-induced propagule formation. However, it would be advantageous to develop additional model systems from early-diverging plant lineages,

as well as charophyte green algae, to study core aspects of plant environmental sensation and responses. For example, the recent genome duplication in *Physcomitrella* has been previously noted (Rensing *et al*., 2007; Rensing *et al*., 2008), and the resulting pairs of paralogs in the genome greatly complements loss-of-function genetics and somewhat undermines *Physcomitrella*'s status as a "haploid system." The work described in Chapter 3 on the CBL-CIPK network in Physcomitrella, wherein CBL and CIPK loci occur as pairs of loci that might be expected to show functional redundancy, illustrates the difficulties that can result. It would be worthwhile to pursue whole-genome sequencing of additional moss species with the aim of identifying a more streamlined genome that does not introduce complications seen in *Physcomitrella*. It remains unclear whether gene targeting is a unique feature of *Physcomitrella*; or whether mosses, or maybe even more distantly related bryophytes, might also be amenable to gene targeting procedures, which may be further refined using genome editing technologies such as the CRISPR-Cas system (Jinek *et al*., 2012).

More than 30 years since seminal observations firmly implicated calcium signaling as a key regulatory mechanism underlying environmental detection and responses in plants (reviewed by Trewavas, 1999; Hepler, 2005), calcium signaling processes remain a vibrant research topic with many questions yet to be answered. As a common output of calcium signaling networks, protein phosphoregulation is an importantly linked process that can connect rapidly occurring signals of the environment to more sustained responses in plants. Prior studies and original research presented here have provided extensive information about calcium-regulated protein kinases in plants. In addition to investigating upstream systems that emit and shape calcium signals, future research should focus on kinase regulatory targets. The responses triggered by signaling outputs of plant calcium-regulated protein kinases can be conceptually categorized according to timescale: short-term or rapid responses achieved by phosphoregulation of ion channels or transporters and long-term responses achieved by activation of transcriptional cascades. This dissertation research contributes to knowledge about both timescales, as phosphoregulation of ion transporters via CBL-CIPK complexes provides an example of rapid responses, whereas the initiation of transcriptional cascades by CYCLOPS in response to phosphorylation by CCaMK exemplifies long-term changes triggered by calcium signals. Looking forward, functional characterization of additional calcium-regulated protein kinases and computational modeling of aggregate outcomes of short- and long-term responses mediated by calcium-decoding protein kinases will be key to advancing our understanding of plant environmental responses.

Sources Cited:

Hepler P. K. (2005). Calcium: a central regulator of plant growth and development. *The Plant Cell* 17, 2142-2155.

Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J. A., & Charpentier E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816-821.

Rensing S.A., Ick J., Fawcett J.A., Lang D., Zimmer A., Van de Peer Y., & Reski R. (2007) An ancient genome duplication contributed to the abundance of metabolic genes in the moss *Physcomitrella patens*. *BMC Evolutionary Biology*, 7, 130.

Rensing S.A., Lang D., Zimmer A.D., Terry A., Salamov A., Shapiro H., …, & Boore J.L. (2008). The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science* 319, 64-69.

Shaw S. L., & Ehrhardt D. W. (2013). Smaller, faster, brighter: advances in optical imaging of living plant cells. *Annual Review of Plant Biology* 64, 351-375.

Swanson S. J., Choi W.-G., Chanoca A., & Gilroy S. (2011). *In vivo* imaging of Ca^{2+}, pH, and reactive oxygen species using fluorescent probes in plants. *Annual Review of Plant Biology* 62, 273-297.

Trewavas A. (1999). How plants learn. *Proceedings of the National Academy of Sciences U.S.A.* 96, 4216-4218.