CHAPTER 13

MscS-Like Proteins in Plants

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I. OVERVIEW

Mechanotransduction is the process by which physical information about the extra- and intracellular environment is converted to a biochemical signal. How plants respond to mechanical stimuli has been under investigation since the work of Darwin (Darwin and Darwin, 1880), but little is

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known about the molecules involved. Response to mechanical stimuli such as gravity, temperature, turgor pressure, and touch are important for plant growth and development. Tension-responsive ion channel activities have been discovered in the plasma and vacuolar membranes of many plant species and plant cell types. However, the molecular identities of these channel activities are not known, nor have they been clearly correlated with a physiological function. Molecular genetic, cell biological, and biochemical approaches are being used in concert with electrophysiology and phylogenetics to characterize a family of putative mechanosensitive (MS) ion channels in the model plant *Arabidopsis thaliana* and evaluate their role as mechanoreceptors in plants.

II. MECHANOSENSATION AND ION CHANNELS IN PLANTS

Animal and bacterial cells sense mechanical stimuli like sound, touch, or osmotic pressure through the action of MS ion channels (Sukharev and Corey, 2004; Kung, 2005; Perozo, 2006). MS ion channels may be activated directly through changes in membrane tension, or indirectly through tethers to the cytoskeleton or extracellular matrix. In either case, the characteristics of the membrane in which MS ion channels are embedded, such as fluidity and curvature, can influence channel activity. The activation of an MS ion channel results in a large but transient flux of ions across a membrane and can lead to rapid changes in cell volume, increased intracellular levels of the second messenger Ca^{2+}, or the production of an electrical current (Kung and Blount, 2004).

A. Plant Cells and Turgor Pressure

It is likely that plants use MS ion channels to mediate mechanosensory events, though the conditions under which they do so would differ from those of animals. Plant cells are surrounded by a thick, semi-rigid wall made of cellulose and other polysaccharides (Cosgrove, 2005). As the plasma membrane is semipermeable, solutes collect inside the protoplast and produce an osmotic potential, which presses the plasma membrane against the cell wall and generates a hydrostatic pressure referred to as turgor. Turgor is important for maintaining plant structure and shape, for cell growth, and for movement (Findlay, 2001). Turgor pressure in a growing epidermal leaf cell is estimated to be as high as 15–20 atm (Pritchard, 2001), and the resting

membrane tension of a plant protoplast is ~0.12 millinewton (mN)/m (Morris and Homann, 2001). Thus, plant plasma membrane systems operate under relatively high pressure, which must influence the way in which plant cells sense and react to both internal and external mechanical stimuli.

B. Mechanosensory Signal Transduction in Plants

Any cellular event that causes membrane deformation or a change in tension, fluidity, or curvature could potentially activate MS ion channels. Turgor pressure, gravity, touch, and temperature are mechanical stimuli whose perception is thought to involve the action of MS ion channels. Below is a brief introduction to the current state of knowledge about plant response to these stimuli, and a review of the data supporting a role for MS ion channels in their perception. For more detailed information about these phenomena, the reader is referred to several excellent reviews (Ramahaleo *et al.*, 1996; Fasano *et al.*, 2002; Braam, 2005; Perrin *et al.*, 2005). MS ion channels may also control events that are less well characterized, such as surface area homeostasis and control of organelle morphology (Raucher and Sheetz, 1999; Morris and Homann, 2001).

1. Osmotic and Turgor Pressure

Plant cells enlarge through enzymatic loosening of the cell wall, followed by turgor-driven expansion of the plasma membrane. Growth at the tips of pollen tubes and root hairs requires a localized gradient of Ca^{2+} ions (Pierson *et al.*, 1994; Bibikova *et al.*, 1997; Felle and Hepler, 1997). It has been proposed that as the cell wall yields during tip growth, turgor pressure deforms the plasma membrane and activates MS ion channels, thereby creating a tip-focused Ca^{2+} gradient (Feijo *et al.*, 2001). MS ion channels may also play a role in the turgor-driven movement of guard cells. Large changes in the volume of guard cells control the opening and closing of stomata, pores through which the plant exchanges water and gas with the environment. Stretch-activated ion channels are thought to function at the level of feedback control of guard cell volume and turgor (Cosgrove and Hedrich, 1991; Grabov and Blatt, 1998; MacRobbie, 1998). MS ion channels may also control cellular volume in response to environmental osmotic stress (Ramahaleo *et al.*, 1996; Shabala and Lew, 2002). Hypoosmotic stress induces the efflux of Cl^- anions from *Arabidopsis* tissue culture cells (Teodoro *et al.*, 1998), and similar results have been obtained with marine algae (Findlay, 2001; Shepherd *et al.*, 2002).

2. Gravity

Higher plants respond to gravity by growing in the appropriate direction with respect to the gravity vector; roots grow down and shoots grow up. Specialized gravity-sensing cells are located in the tip of the root and in the starch-sheath layer, or endodermis, of the stem. These cells contain dense, starch-filled plastids termed amyloplasts. Substantial experimental data supports a model wherein movement (but not necessarily sedimentation) of amyloplasts in response to changes in the gravity vector provides the gravitropic signal (Kiss, 2000; Perrin *et al*., 2005). There is also evidence for an amyloplast-independent mechanism of gravity signaling; in this case, the entire weight of the protoplasm may provide the directional signal (Kiss *et al*., 1989; Staves, 1997).

Gravity perception in both green algae and vascular plants has long been hypothesized to involve MS ion channels (Sievers, 1991; Ding and Pickard, 1993a; Yoder *et al*., 2001; Blancaflor and Masson, 2003). Amyloplast movement within the cytosol may disrupt the actin cytoskeleton and thereby transmit a signal to MS ion channels located in the plasma membrane (Blancaflor, 2002; Palmieri and Kiss, 2005). However, little experimental data exists to directly link ion channel activity and the early stages of gravity perception. Gravity stimulation of *Arabidopsis* roots is correlated with the rapid alkalinization of the cytosol and concomitant acidification of the extracellular space in the root cap, and preventing this pH change with the use of caged protons delays the gravitropic response (Scott and Allen, 1999; Fasano *et al*., 2001; Johannes *et al*., 2001). The cause and effect of the observed cytoplasmic pH changes in the columella cells of the root tip is not yet clear.

3. Temperature

Plants acclimate to the cold in a process that involves induction of cold-responsive genes, accumulation of cryoprotectants, and modification of membrane composition (Thomashow, 1999). It is not known how a change in temperature is first perceived, but MS ion channels that are responsive to membrane fluidity have been proposed to fill this role. Experiments utilizing a luminescent Ca^{2+} reporter show a transient Ca^{2+} influx into the cytosol during cold acclimation (Knight *et al*., 1991), and preventing this influx with Ca^{2+} channel blockers or inhibitors of Ca^{2+}-binding proteins also prevents cold acclimation and the induction of cold-responsive genes (Monroy *et al*., 1993; Tahtiharju *et al*., 1997). Evidence that this Ca^{2+} influx is due to a mechanically gated ion channel comes from experiments with alfalfa cell suspension cultures, where artificial fluidization of the membrane prevents both cold-induced Ca^{2+} influx and cold acclimation. Conversely, membrane

rigidification activates cold-signaling pathways in cells incubated at room temperature (Orvar *et al.*, 2000).

4. Touch

Plants show a wide variety of responses to touch (Jaffe *et al.*, 2002; Braam, 2005). Touch can induce a fast movement, like the shutting of a Venus flytrap, or a tropic response, as in the twining of a pea vine. Further, plants respond to repeated touch or wind by changes in growth rate and in morphology (in general, a reduced stem height and increased girth). At the cellular level, pressure can cause alterations in the cell division plane (Lintilhac and Vesecky, 1984), and the migration of chloroplasts and nuclei within the cell (Kennard and Cleary, 1997; Sato *et al.*, 1999). A number of experiments implicate Ca^{2+} ion transients in these touch responses. Cytoplasmic Ca^{2+} levels increase immediately in tobacco seedlings stimulated by a puff of air blown through a syringe in protoplasts swirled in solution, and in *Arabidopsis* root cells touched with a glass capillary (Knight *et al.*, 1992; Haley *et al.*, 1995; Legue *et al.*, 1997). *Chara* cells respond to touch by rapid depolarization of the membrane and a subsequent increase in cytoplasmic Ca^{2+} (Shepherd *et al.*, 2002, and references therein).

C. MS Ion Channels Are Present in Plant Cell Membranes

Over the last 20 years, the electrophysiological method of patch clamping has been used to identify and characterize distinct MS ion channel activities in a variety of plant species and cell types (Falke *et al.*, 1988; Schroeder and Hedrich, 1989; Alexandre and Lassalles, 1991; Cosgrove and Hedrich, 1991; Badot *et al.*, 1992; Ding and Pickard, 1993a; Spalding and Goldsmith, 1993; Garrill *et al.*, 1994; Moran *et al.*, 1996; Lewis and Spalding, 1998; Liu and Luan, 1998; Yoshimura, 1998; Heidecker *et al.*, 1999; Dutta and Robinson, 2004; Qi *et al.*, 2004). Some of these activities are found in cell types involved in plant movements, such as guard cells and the leaflet motor cells that control the circadian leaf movements of legumes. These channel activities and their characteristics are listed in Table I. Most were found in the plasma membrane, though two were found in vacuolar membranes. The channels range from nonselective to selective, and their conductance range from 3 to 100 picosiemens (pS), similar to the conductance of other ion channels observed in plant membranes (White, 1998; Demidchik *et al.*, 2002). A hallmark of MS ion channels is patch fatigue or adaptation (Hamill and Martinac, 2001). Activities found in both onion bulb cell vacuoles and plasma membranes,

TABLE I

Mechanosensitive Ion Channel Activities in Plant Membranes

Plant and cell type	Activation pressure	Patch type	Channel type	Conductance (conditions)[a]	Notable characteristics	References
Nicotiana tabaccum suspension cell culture	Negative	Inside-out	Anion	97 pS (220 Cl^-: 25 Cl^-)		Falke *et al.*, 1988
Commelina communis guard cells	Negative	Outside-out	ND	ND	Two conductance states observed	Schroeder and Hedrich, 1989
Beta vulgaris root cell vacuoles	Positive or negative	Outside-out	Nonselective	20 pS (200 K^+, 204 Cl^-: 200 K^+, 206 Cl^-, 1 Ca^{2+})	Inhibited by Gd^{3+}, activated by hyper- and hypoosmotic gradients	Alexandre and Lassalles, 1991
Vicia faba guard cells	Negative	Outside-out	Cl^-	27 pS[b](150 Cl^-: 40 Cl^-)		Cosgrove and Hedrich, 1991
	Negative	Outside-out	K^+	45–50 pS[b] (150 K^+: 24 K^+)		
	Negative	Outside-out	Ca^{2+}	3 pS (150 K^+: 30 Ca^{2+})		
Allium cepa parenchyma cell vacuoles	Negative	Cell-attached	ND	ND	Evidence for linked conductance units	Badot *et al.*, 1992
A. thaliana leaf mesophyll	Negative	Inside-out	Nonselective	ND		Spalding and Goldsmith, 1993

Allium cepa leaf sheath epidermis	Negative	Inside-out	Ca^{2+} K^+	6.5 pS(200 Ca^{2+}: 100 Ca^{2+})	Inhibited by Gd^{3+}, linked conductance units, sensitive to temperature	Ding and Pickard, 1993a,b; Pickard and Ding, 1993
Zostera muelleri epidermis	Negative	Outside-out	K^+	100 pS (140 K^+, 2.3 Ca^{2+}, 8.6 Cl^-: 110 K^+, 10 Ca^{2+}, 20 Cl^-)	Whole-cell action potentials were affected by osmolarity and inhibited by Gd^{3+}	Garrill *et al.*, 1994
Samanea saman leaflet motor cells	Positive	Outside-out	ND	5.2 pS (131 K^+, 0.4 Ca^{2+}, 126 Cl^-: 55 K^+, 0.5 Ca^{2+}, 1 Cl^-)		Moran *et al.*, 1996
Vicia faba guard cells	Negative	Outside-out	K^+	95 pS[b](100 K^+ symmetric)	Not affected by osmotic gradient	Liu and Luan, 1998
Arabidopsis thaliana etiolated hypocotyl	Negative	Outside-out	Nonselective	39 pS (138 Cl^-, 1.9 Ca^{2+}: 130 K^+)		Lewis and Spalding, 1998
Chlamydomonas reinhardtii	Negative	Cell-attached	ND	30–50 pS (1 K^+, 1.3 Cl^-, 0.3 Ca^{2+} symmetric)	Inhibited by Gd^{3+}; similar to channel found in the flagella	Yoshimura, 1998
Valonia utricularis mother cells	Negative	Cell-attached	Cl^-	22 pS (150 Cl^-: 30 Cl^-)		Heidecker *et al.*, 1999

(*Continued*)

TABLE I (*Continued*)

Plant and cell type	Activation pressure	Patch type	Channel type	Conductance (conditions)[a]	Notable characteristics	References
Arabidopsis thaliana leaf mesophyll	Positive	Outside-out	Anion	100 pS[c] (20 Cl^-: 80 Cl^-)	Negative pressure has no effect; activated by the amphipath TNP	Qi *et al.*, 2004
Lilium longiflorum pollen grain	Negative	Outside-out	K^+	33 pS (150 K^+: 24 K^+)	Inhibited by Gd^{3+} and by spider venom; channels localize to domains on surface of grain and tube protoplasts	Dutta and Robinson, 2004
Lilium longiflorum pollen grain and pollen tube	Negative	Outside-out	Ca^{2+}	15 pS (150 K^+: 30 Ca^{2+})		

[a]Pipette solution: bath solution (in mM).
[b]Outward conductance.
[c]From −100 to 100 mV, calculated from published current–voltage curve.
ND, not determined.

as well as in *Arabidopsis* mesophyll and pollen protoplasts have this behavior. Table I lists only those activities identified through patch clamping; other techniques to analyze ion flux have also provided evidence for the presence of MS ion channels in plant membranes (e.g., Teodoro *et al*., 1998; Kikuyama and Tazawa, 2001).

To summarize, it is clear that plants respond to a number of mechanical stimuli and MS ion channel activities are present in their membranes. The challenge now is to determine if these observed activities are responsible for the perception of mechanical stimuli. Treatment with gadolinium (Gd^{3+}) ions and spider venom, known to inhibit MS ion channels (Yang and Sachs, 1989; Chen *et al*., 1996), can also prevent many of the mechanosensory responses described above, including pollen tube growth, root and hypocotyl gravitropism, cold acclimation, and chloroplast migration (Ding and Pickard, 1993a; Tahtiharju *et al*., 1997; Sato *et al*., 2003; Dutta and Robinson, 2004; Soga *et al*., 2004). However, the Ca^{2+} increase observed in response to wind is insensitive to treatment with Gd^{3+} (Knight *et al*., 1992), and the specificity of gadolinium inhibition in plant cells has been questioned (White, 1998). Definitively linking plant mechanosensory perception and MS ion channels will require the use of multiple techniques in concert, including electrophysiology, cell physiology, and genetics. One area where this approach is underway is the study of a family of plant proteins related to the bacterial MS ion channel protein MscS.

III. THE EUKARYOTIC FAMILY OF MscS-LIKE PROTEINS

A. E. coli *MscS*

1. Introduction

The electrophysiological activity that has become known as MscS (*M*echano-*s*ensitive *c*hannel, *S*mall conductance) was first identified during electrophysiological characterizations of the plasma membrane of giant *Escherichia coli* spheroplasts (Martinac *et al*., 1987; Sukharev *et al*., 1993; Cui *et al*., 1995; Berrier *et al*., 1996). MscS has a conductance of 350–950 pS, depending on the ionic conditions, and is largely nonselective. The open probability of MscS is increased by negative pressure (suction) in a voltage-modulated manner and can also be activated by an osmotic gradient (Cui *et al*., 1995). Like other stretch-activated ion channels, MscS is reversibly inhibited by Gd^{3+} ions (Berrier *et al*., 1992).

YggB, the gene responsible for MscS activity in *E. coli*, was cloned and its product appears to function as an osmotic safety valve, helping to prevent

cellular rupture during hypoosmotic shock (Levina *et al*., 1999). The molecular identification of MscS allowed controlled purification and reconstitution experiments, which demonstrated that MscS is directly responsive to membrane tension, and does not require any other cellular structures for its gating function (Okada *et al*., 2002; Sukharev, 2002). The crystal structure of *E. coli* MscS revealed a homoheptamer, with three TM domains contributed by each monomer and a large C-terminal chamber, thought to serve as a prefilter (Bass *et al*., 2002). This structure features an open pore of ~11 Å, though whether it represents the native open structure is a topic of current discussion (Perozo, 2006).

2. Important Features of MscS Sequence

Several important features of the primary sequence of MscS have been identified through mutant analysis and inspection of the crystal structure. The pore-lining transmembrane (TM) domain, TM3, contains a repeating pattern of glycine and alanine residues that is similar to that found in the pore-lining TM of another MS ion channel from bacteria, MscL-, and MscS-related proteins in the archaeon *Methanococcus jannaschii* (Kloda and Martinac, 2001a).The introduction of bulky hydrophobic residues within TM3 at G104, A106, and G108 subtly increases the amount of pressure required to gate the channel (Edwards *et al*., 2005), while substitution of proline for A102 causes a strong gain-of-function (GOF) phenotype (Miller *et al*., 2003a). These results are consistent with a model wherein close packing of the TM3 residues is required both for maintaining a closed pore and for transitioning to the open state. Other important residues are L105 and L109, predicted to create a narrowing of the channel at the cytoplasmic surface, referred to as the hydrophobic seal. Replacing L109 with serine causes a strong GOF phenotype (Miller *et al*., 2003a). Three arginine residues embedded in TM1 and TM2 may contribute to voltage sensitivity in bacterial and archaeal MscS homologues (Kloda and Martinac, 2001a; Bass *et al*., 2002; Edwards *et al*., 2004).

Also conserved among MscS family members is the sequence located directly C-terminal (cytoplasmic in MscS) to the pore-lining TM3 (Pivetti *et al*., 2003), though no role has been attributed specifically to the conserved domain. The entire C-terminal domain is important for stability and activity and appears to undergo a large rearrangement on opening of the pore (Koprowski and Kubalski, 2003; Miller *et al*., 2003b; Schumann *et al*., 2004). The C-terminal domain may also be involved in contact with intracellular regulators. PamA, an MscS family member from *Synechocystis*, interacts with a signaling protein involved in carbon and nitrogen metabolism both *in vitro* and in the yeast two-hybrid assay (Osanai *et al*., 2005).

B. The Eukaryotic Subfamily

1. Overview

Phylogenetic analyses have identified members of the MscS family of MS ion channels in bacterial and archaeal species, in fission yeast, and in *Arabidopsis* (Koprowski and Kubalski, 2001; Kloda and Martinac, 2002; Pivetti *et al*., 2003). Most genomes contain multiple MscS paralogues (e.g., six MscS-like proteins were identified in *E. coli*), suggesting multiple functional roles for this family of proteins. A more directed investigation into the eukaryotic branch of this family identified genes encoding MscS-like proteins in organisms from three of the four eukaryotic kingdoms: plants, fungi, and protists. Multiple *MscS-like* (*MSL*) genes were found in each of the four plant genomes that have been sequenced: 10 in *A. thaliana* (mouse-ear cress), 4 in *Chlamydomonas reinhardtii* (green algae), 6 in *Oryza sativa* (rice), and at least 7 in *Populus trichocarpa* (poplar tree). These genes are listed in Table II. In addition, expressed sequence tags (ESTs) or cDNAs containing related sequence have been reported in agriculturally relevant plants such as wheat, corn, tomato, and sorghum. Genes predicted to encode MscS-like proteins were also found in the cellular slime mold *Dictyostelium discoideum* and in several fungal organisms, including fission yeast (*Schizosaccharomyces pombe*), several species of *Aspergillus*, and *Neurospora crassa*. However, it appears that there are no *MSL* genes in the genome of the budding yeast *Saccharomyces cerevisiae* nor were any MscS-like proteins that conform to the consensus sequences shown in Fig. 1 found encoded in any animal genomes (but see Koprowski and Kubalski, 2001).

2. Class I and Class II Proteins

The eukaryotic members of the MscS family can be organized into two main classes based on sequence similarity (Fig. 1). In agreement with a previous analysis by Pivetti *et al.* (2003), the conserved motif includes the most C-terminal TM domain and surrounding sequence. Class I proteins align relatively closely with *E. coli* MscS (Fig. 1A). Proteins in this group all have C-terminal TM domains that resemble TM3 of MscS in that they are rich in glycine and alanine residues, though the pattern is not conserved. The hydrophobic seal residues of MscS, L105 and L109, align with bulky hydrophobics (V, L, or F) in the eukaryotic Class I proteins. C-terminal to the TM domain is the consensus sequence $PF(X_{12–16})GXV(X_{20–21})PN(X_9)N$. This sequence is related to the MscS family consensus sequence identified by Pivetti *et al.* (2003), as shown in Fig. 1A.

The C-terminal TM domain of Class II proteins is not glycine- or alanine-rich like that of MscS and the Class I proteins, but contains amino acids with

TABLE II

MscS-Like Proteins in Eukaryotes

Organism	Name or UniProt code	Class	Predicted subcellular localization (predotar)	Score
Plants				
A. thaliana	MSL1 (At4g00290)	I	Mitochondria	0.48
A. thaliana	MSL2 (At5g10490)	I	Plastid	0.39
A. thaliana	MSL3 (At1g58200)	I	Plastid	0.16
A. thaliana	MSL4 (At1g53470)	II	Elsewhere	0.99
A. thaliana	MSL5 (At3g14810)	II	Elsewhere	0.99
A. thaliana	MSL6 (At1g78610)	II	Elsewhere	0.99
A. thaliana	MSL7 (At2g17000)	II	Elsewhere	0.97
A. thaliana	MSL8 (At2g17010)	II	Elsewhere	0.97
A. thaliana	MSL9 (At5g19520)	II	Elsewhere	0.99
A. thaliana	MSL10 (At5g12080)	II	Elsewhere	0.99
Beta vulgaris	Q1ZY11	II	[b]	
Brachypodium sylvaticum	Q2L3D9	II	[a]	
Brassica campestris	Q4ABZ2	I	Plastid	0.19
C. reinhardtii	MSC1 (Clre: 144381)	I	Elsewhere	0.99
C. reinhardtii	MSC2 (Clre: 146627)	I	Elsewhere	0.95
C. reinhardtii	MSC3 (Clre: 146630)	I	Plastid	0.33
C. reinhardtii	MSCL4 (Chlre: 178587)	I	Mitochondria	0.32
C. reinhardtii	MSCL1 (Chlre: 143022)	II	Elsewhere	0.99
Lycopersicon esculentum	Q949J9	I	Plastid	0.51

O. sativa	Os02g45690	I	Mitochondria	0.76
O. sativa	Os04g48490	I	Mitochondria	0.17
O. sativa	Os03g31850	I	Plastid	0.95
O. sativa	Os02g44770	II	Elsewhere	0.96
O. sativa	Os04g47320	II	Elsewhere	0.99
O. sativa	Os06g10410	II	Elsewhere	0.88
P. trichocarpa	Poptr1: 347999	I	Mitochondria	0.66
P. trichocarpa	Poptr1: 572294	I	Mitochondria	0.73
P. trichocarpa	Poptr1: 588141	I	Mitochondria	0.13
P. trichocarpa	Poptr1: 563295	I	[a]	
P. trichocarpa	Poptr1: 546736	II	Elsewhere	0.98
P. trichocarpa	Poptr1: 589266	II	Elsewhere	0.99
P. trichocarpa	Poptr1: 352620	II	Elsewhere	0.95
Sorghum bicolor	Q8XOU2	II	Elsewhere	0.99
Triticum aestivum	Q2L3W4	II	[a]	
Triticum aestivum	Q2L3UB8	II	[a]	
Zea mays	Q6QP48	II	Elsewhere	0.99
Zea mays	Q6QP53	II	Elsewhere	0.98
Fungi				
Aspergillus fumigatus	Q4X1L2	II	Elsewhere	0.90
Aspergillus fumigatus	Q4X020	II	Elsewhere	0.99
Aspergillus nidulans	Q5B077	II	Elsewhere	0.99
Aspergillus nidulans	Q5AVV9	II	Elsewhere	0.99
Aspergillus oryzae	Q2TZV3	II	Elsewhere	0.90
Aspergillus oryzae	Q2UCW6	II	Elsewhere	0.99

(*Continued*)

TABLE II (*Continued*)

Organism	Name or UniProt code	Class	Predicted subcellular localization (predotar)	Score
Chaetomium globosum	Q2HBD6	II	Elsewhere	0.99
Neurospora crassa	Q8NIY0	II	Elsewhere	0.99
Neurospora crassa	Q873L5	II	Elsewhere	0.99
Schizosaccharomyces pombe	SPO2 (O74839)	II	Elsewhere	0.99
Protists				
Dictyostelium discoidium	Q86AP5	II	[b]	

[a]Sequence fragment.
[b]Low quality sequence.

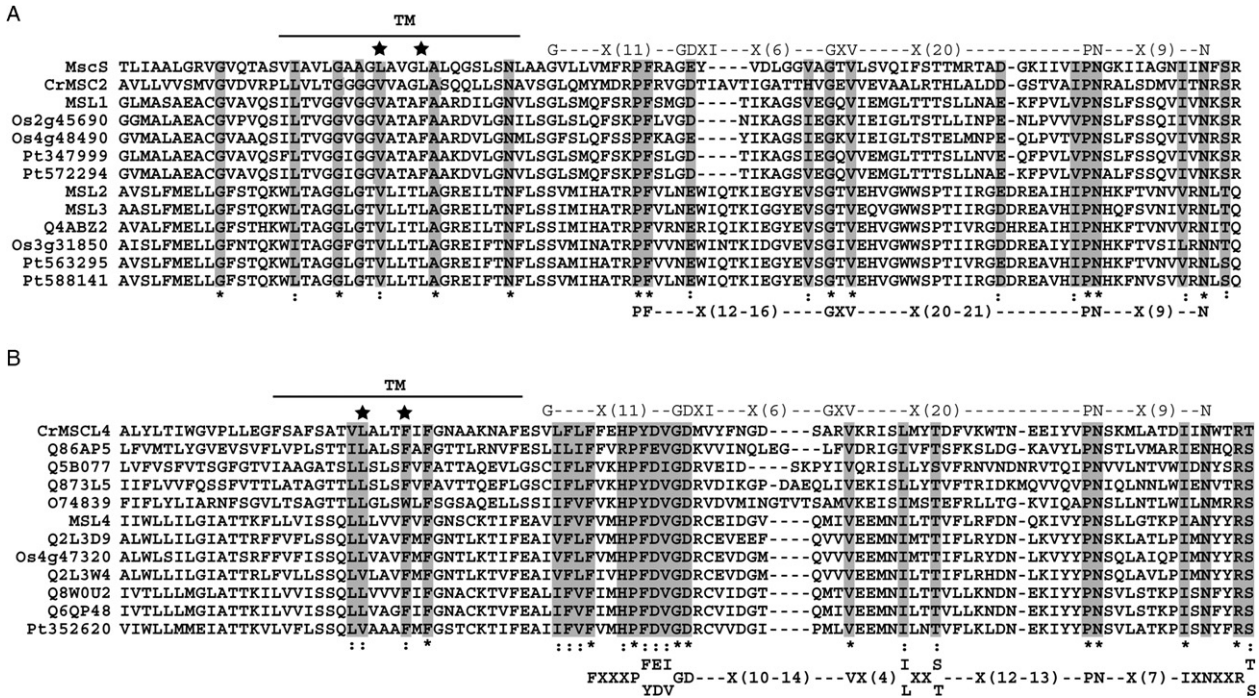

FIGURE 1 MscS family conserved domains. Amino acid sequence of MscS family members from *E. coli* (MscS); *C. reinhardtii* (CrMSC2, CrMSC4); *A. thaliana* (MSL1, MSL2, MSL3, MSL4); *Brachypodium sylvaticum* (Q2L3D9); *Brassica campestris* (Q4ABZ2); *O. sativa* (Os2g45690, Os4g48490, Os3g31850, Os4g47320); *P. trichocarpa* (Pt345999, Pt572294, Pt563295, Pt588141, Pt352620); *Sorghum bicolor* (Q8XOU2); *Triticum aestivum* (Q2L3W4); *Zea mays* (Q6QP48); *Aspergillus nidulans* (Q5B077); *D. discoidium* (Q86AP5); *Neurospora crassa* (Q873L5); and *Schizosaccharomyces pombe* (SPO). Alignment was performed using ClustalX. Identical (*) and similar (.) residues are

larger hydrophobic side chains. Large hydrophobic amino acids are conserved at certain positions within the domain (marked with stars in Fig. 1B); whether these correspond to hydrophobic seal residues is not yet known. The proteins in Class II contain the consensus sequence $F(X_3)P(X_3)GD(X_{10-14})V(X_{20-21})PN(X_7)IXNXXR$. Class II contains those proteins designated as Group XVII by Pivetti *et al.* (2003), and is related to their consensus sequence as shown in Fig. 1B.

Though assigned by sequence alignment, Class I and Class II proteins also differ in the cellular compartment in which they reside and the organisms in which they are found. All 15 Class I proteins shown in Table I are from plants, and 13 of these are predicted to localize to mitochondria or to chloroplasts. The exceptions are two MscS homologues from *Chlamydomonas*, MSC1 and MSC2, which are not predicted to localize to endosymbiotic organelles. It is possible that the N-terminal sequences of MSC1 and MSC2 are incorrectly annotated, or that they contain cryptic transit peptides. Alternatively, some members of Class I may not localize to organelles. In contrast, Class II proteins are found in organisms as diverse as plants, fungi, and *Dictyostelium*, and do not contain organelle-targeting sequences. It thus appears that the two classes of eukaryotic MscS homologues have evolved to function in different cellular compartments, and that Class I but not Class II proteins are restricted to the plant lineage. The potential evolutionary implications of these observations are discussed at the end of this chapter.

3. Plant MscS-Like Proteins Are Likely to Form MS Ion Channels

MscS-related proteins are good candidates for the molecules underlying the MS ion channel activities that have been previously observed in plant membranes. Indeed, many of these activities, listed in Table I, share several characteristics with *E. coli* MscS homologue (summarized in Sukharev *et al.*, 1997), and may therefore be provided by a plant MscS homologue. Though MscS is slightly anion selective, MscMJ, a homologue from archaea, prefers cations (Kloda and Martinac, 2001b), making it impossible to predict the ion-specificity of other MscS-like channels. The two anion-selective ion channels described in tobacco and *Arabidopsis* plasma membranes have relatively large conductance (~100 pS), though still tenfold lower than that of MscS.

indicated at the bottom, both are shaded. Consensus sequences derived from this analysis is presented at the bottom of each alignment in dark type; the consensus sequence derived by Pivetti *et al.* (2003) is at the top and of each alignment in gray type. (A) Class I proteins. Filled-in stars indicate predicted hydrophobic seal residues, while the open star marks G113. The line indicates the experimentally derived MscS TM3 domain. (B) Representative Class II proteins. The line indicates the location of a consensus TM domain according to the Aramemnon database.

In most cases, pressure-sensitive activity is seen in excised patches, demonstrating that these channels can open without an extended interaction with other cellular components, as can MscS. Further, the activities in beet vacuoles and in sea grass epidermal cells are activated by the introduction of an osmotic gradient, and the channel in *Arabidopsis* mesophyll cells is activated by the incorporation of trinitrophenol, which induces an increase in membrane curvature through insertion into the outer leaflet. These data suggest that membrane deformation is the primary stimulus required for gating of these channels.

One way to attribute MS ion channel activity to an MscS-like protein is to determine if an activity observed in wild-type membranes, such as those described above, is compromised in plants harboring mutations in MscS-related genes. To date, results for such an experiment have not been reported. However, evidence that eukaryotic members of the MscS family can function as MS ion channels has been provided by heterologous expression experiments. Two Class I *Arabidopsis* proteins, MSL3 and MSL1, can provide protection from hypoosmotic shock in an *E. coli* strain lacking MS ion channel activity (Haswell and Meyerowitz, unpublished; Haswell and Meyerowitz, 2006). Though these results are consistent with the hypothesis that the MSL proteins form MS ion channels, electrophysiological experiments can more directly assess this proposal. Preliminary patch-clamp data suggest that an MscS family member from *Chlamydomonas* can provide MS ion channel activity when expressed in giant *E. coli* spheroplasts (K. Yoshimura, personal communication). It thus seems likely, though is not yet established, that the eukaryotic subfamily of *MSL* genes encodes ion channels that are mechanically gated.

IV. THE *ARABIDOPSIS MSL* GENES

A. Overview

To determine if MscS-like ion channels are involved in plant mechanosensation, we have begun to characterize a family of MscS-related proteins in the model plant *A. thaliana*. A small flowering plant of the mustard family, *Arabidopsis*, has been widely used in studies of mechanosensation and electrophysiology and has the advantages of a sequenced genome and many publicly available genomic and proteomic tools. As shown in Table II, there are 10 *MSL* genes in the *Arabidopsis* genome: *MSL1–3* encode Class I proteins, and *MSL4–10* encode proteins belonging to Class II. An 11th gene, At4g00234, is closely related (96% identical at amino acid level) and physically linked to *MSL1*, but lacks the last TM domain and the MscS consensus sequence.

An unrooted phylogenetic tree illustrating the evolutionary relationship between the *Arabidopsis* MSL proteins and *E. coli* MscS is shown in Fig. 2. This tree was built with the conserved amino acid sequence in Fig. 1A. This phylogram illustrates two points about the *Arabidopsis* MSL proteins. First, several members of the family are highly similar, suggesting that they may function redundantly. MSL2 and MSL3 are 50% identical and mutant analysis indicates that they are indeed redundant (see below). It is, therefore, likely that other MSL proteins are functionally redundant. *MSL4* and *MSL5* are products of the most recent *Arabidopsis* genome duplication (Blanc *et al.*, 2003)

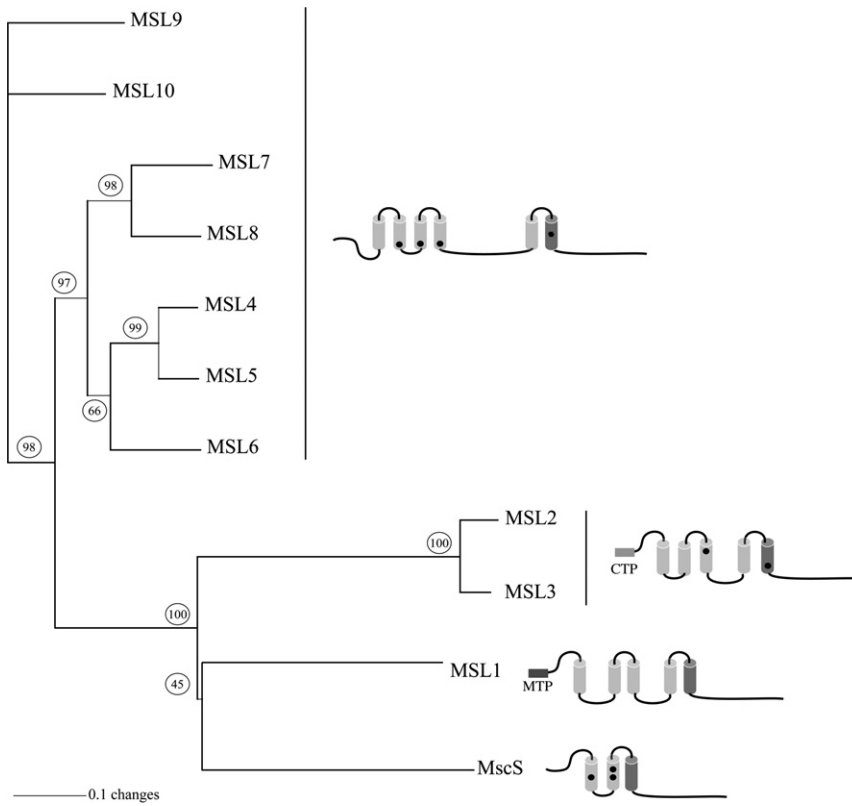

FIGURE 2 Phylogenetic tree of the *Arabidopsis* MSL protein family. The most C-terminal TM domain and adjacent sequence was aligned in ClustalX (as in Fig. 1A). Then PAUP was used to generate an unrooted neighbor joining tree. Numbers in circles indicate the bootstrap value of each node as a percentage of 1000 replicates. The predicted topologies of the MSL proteins are shown to the right of the tree. Cylinders indicate predicted TM domains. Dots mark the site of basic amino acids or motifs within the predicted TM domains. MTP, mitochondrial transit sequence; CTP, chloroplast transit sequence.

and the proteins that they encode are 68% identical. *MSL7* and *MSL8* encode proteins that are 71% identical, and may also be the result of a duplication, as they are located in tandem on the second chromosome. The second point is that the *MSL* proteins cluster into two general groups, corresponding to Class I and Class II proteins. MSL1, MSL2, and MSL3 cluster with MscS, and by sequence similarity are assigned to Class I. The other seven, MSL4–10, are in a second cluster and are assigned to Class II. Experimental evidence that the Class I/Class II division is biologically relevant is described below in the Section IV.B.

Figure 2 also illustrates the predicted topologies of the MSL proteins (the Aramemnon database; Schwacke *et al.*, 2003). TM3 of MscS and the related C-terminal TM domain from each MSL protein are indicated in dark gray. MSL1, MSL2, and MSL3 have five predicted TM domains, and the consensus prediction for MSL4–10 is for six TM domains. It has been experimentally determined that the C-terminal domain of MscS is located in the cytoplasm (Miller *et al.*, 2003a). Assuming that the large C-terminal domains of MSL4–10 proteins are also in the cytoplasm, their N-termini will also be inside the cytoplasm. Whether the C-terminus of MSL1, MSL2, or MSL3 should be in the cytoplasm or in the organelle lumen is not clear, but could be tested by proteolytic analysis of purified organelles.

The putative ion channels produced by the MSL proteins may be voltage modulated, as several of their TM domains contain basic amino acids. Basic amino acids or motifs are indicated with black dots in Fig. 2. MSL1 does not have basic residues in any of its predicted TM domains, while MSL2 and MSL3 both contain arginine residues at the edges of their third and fifth TM domains. In MSL4–10, conserved motifs containing basic residues can be found at the cytoplasmic edge of predicted TM2 (K/R/H-X2-V), TM3 (R/KKXVQ), and TM4 (KT-X3-K) and a conserved lysine residue is present in the middle of TM6. The motifs found in TM3, TM4, and TM6 are conserved in Class II proteins from rice and maize.

B. Subcellular Localization of MSL Proteins

Consistent with their assignment to Class I of eukaryotic MscS-like proteins, MSL1–3 contain putative N-terminal organelle localization sequences. MSL1 is predicted to localize to mitochondria and MSL2 and MSL3 to plastids, according to the subcellular localization prediction program Predotar (Small *et al.*, 2004), and experimental evidence supports these predictions. Both cell fractionation and live imaging experiments have shown that MSL2-GFP and MSL3-GFP fusions are localized to the plastid envelope (Fig. 4C and Haswell and Meyerowitz, 2006). In *Arabidopsis* root hairs, an MSL1-GFP fusion

protein colocalizes with the mitochondrial marker MitoTracker (Haswell and Meyerowitz, unpublished).

Class II proteins do not contain identifiable localization sequences, and the subcellular localizations of MSL4–10 remain unresolved. MSL10 was identified in a proteomic analysis of the proteins of the vacuolar membrane from suspension-cultured *Arabidopsis* cells (Shimaoka *et al.*, 2004). However, MSL10, along with MSL6, was also identified in an analysis of phosphorylated plasma membrane proteins (Nuhse *et al.*, 2004). Preliminary data suggests that MSL4 is localized to the plasma membrane as a GFP fusion protein (Haswell and Meyerowitz, unpublished data). Clearly, assigning the proper subcellular location for each MSL protein will require further experimentation.

C. *Control of* MSL *Gene Expression*

Results from the GeneAtlas microarray database (Zimmermann *et al.*, 2004) characterizing the tissue-specific expression of six of the ten *MSL* genes are summarized in Fig. 3A. These results, combined with RT-PCR analysis (Fig. 3B and C), demonstrate that the *Arabidopsis MSL* genes are expressed in a variety of tissues throughout the life of the plant. Both approaches show that most of the *MSL* genes are expressed at detectable levels in all the major tissues of the plant. The exceptions are *MSL7*, which is detectable at low levels only in the flower, *MSL9*, which is expressed at high levels but is restricted to the root, and *MSL8*, whose transcripts were not detected by either method. GUS reporter lines provide further refinement of the expression patterns and suggest that *MSL* genes are expressed in specific cell types, including the vasculature, stigma cells of the carpel, and guard cells (Haswell and Meyerowitz, unpublished).

In *E. coli*, MscS channel protein expression is controlled at the transcriptional level by RpoS sigma factor, and is activated by high osmolarity and on entry into stationary phase (Stokes *et al.*, 2003). Environmental and developmental factors also modulate the expression of the *Arabidopsis MSL* genes. According to the RNA-profiling database Genevestigator (Zimmermann *et al.*, 2004), hormone and stress treatments affect the transcript levels of several of the *MSL* genes. For example, *MSL6* transcript levels increase in response to treatment with the plant hormone abscisic acid and to osmotic and salt stress treatments. Not all *MSL* genes behave in a similar fashion, as *MSL9* expression is repressed rather than induced by osmotic stress, and *MSL3* transcripts are increased by treatment with a different plant hormone, methyl jasmonate. *MSL9* was also identified in a microarray analysis of genes that respond to mechanical and gravity stimuli (Kimbrough *et al.*, 2004). It will be important to validate microarray expression results with independent

A

GeneAtlas Expression Data

Tissue Type	MSL1	MSL2	MSL3	MSL6	MSL7	MSL9	MSL10
Suspension cells	++	++	+++	++	-	++	+++++
Seedling	+++	++	++	++	-	++	++
Inflorescence	++++	+	++	++	+	++	+++
Rosette	+++++	++	++	++	-	-	++
Root	+++	++	++	++	-	+++++	+++

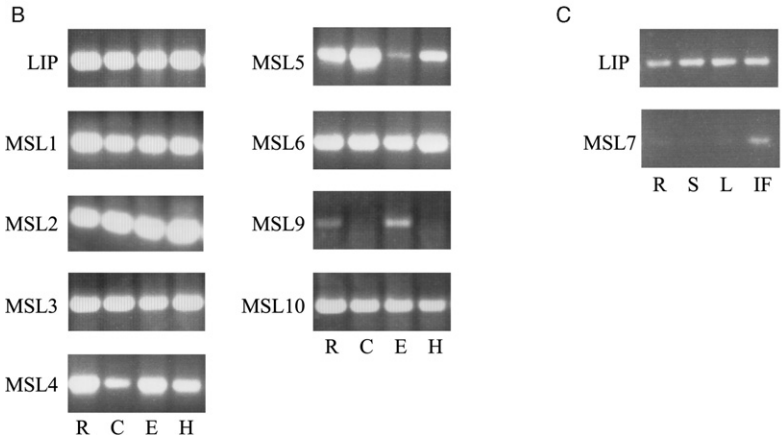

FIGURE 3 Tissue-specific expression of the *MSL* genes. (A) GeneAtlas results. The number of + symbols indicates the relative mean signal strength for each tissue type. (B) and (C) Semiquantitative RT-PCR analysis of *MSL* gene expression in *Arabidopsis* tissues. RNA was isolated from R, root; C, cotyledon; E, etiolated root; H, etiolated hypocotyl; S, stem; L, leaf; IF, inflorescence. LIP, lipase control.

methods, as information about the transcriptional control of the *MSL* genes may provide clues to the function of the proteins they encode.

D. MSL2, MSL3, and the Control of Organelle Morphology

MSL2 and *MSL3* are the only members of the *MSL* gene family about which functional information has been published. Plants harboring insertional mutations in both *MSL2* (*msl2–1*) and *MSL3* (*msl3–1*) show morphological defects in plastids, the plant-specific endosymbiotic organelles responsible for photosynthesis, gravity perception, and numerous metabolic reactions (Haswell and Meyerowitz, 2006). In *msl2–1; msl3–1* double mutants, chloroplasts are normally developed but greatly enlarged, while nonphotosynthetic

plastids are enlarged and abnormally shaped (Fig. 4A and B). Both cell fractionation and live imaging studies demonstrate that MSL2-GFP and MSL3-GFP fusion proteins are localized to plastids, consistent with their assignment as Class I proteins. These GFP-fusion proteins are likely to be functional, as the transgenes that encode them can rescue the *msl2–1; msl3–1* variegated phenotype. MSL2-GFP and MSL3-GFP localize to discrete foci at the poles of all types of plastids (Fig. 4C). As mentioned above, MSL3 can rescue the osmotic shock sensitivity of an *E. coli* mutant lacking MS ion activity, implying that MSL3 does indeed function as an MS ion channel.

Organelle morphology in plants is highly dynamic and is presumably controlled by a variety of mechanisms. For example, large changes in vacuolar morphology accompany the opening of guard cells in *Vicia faba* (Gao *et al*., 2005). During the early stages of tobacco leaf cell protoplast production (de-differentiation), individual mitochondria undergo a fusion process to produce a large tubular network (Sheahan *et al*., 2005). Chloroplast size is under active control, as 11 *accumulation and replication of chloroplasts* (*arc*) mutants have been identified that exhibit changes in chloroplast size and number (summarized in Aldridge *et al*., 2005).

If MSL2 and MSL3 form MS ion channels in the plastid envelope, as predicted, what is their role there? The preliminary characterization of the *msl2–1; msl3–1* mutant phenotype suggests a number of answers to this question. One proposal is that MSL2 and MSL3 (MSL2/3) play a role in controlling the number and size of chloroplasts in each cell. MS ion channels located in the plastid envelope might sense the pressure generated by tight packing of chloroplasts and activate plastid division when the pressure

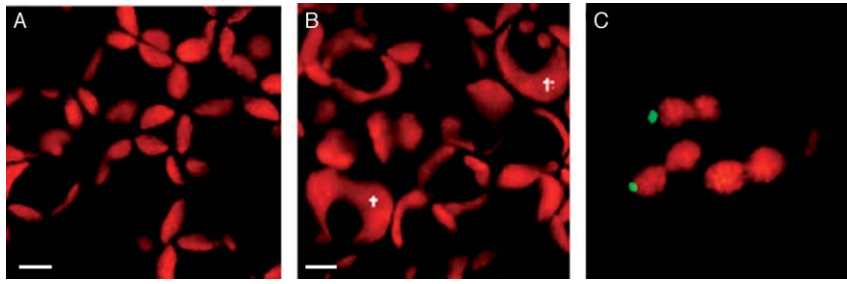

FIGURE 4 (A) Confocal microscopy of chloroplasts in the mesophyll cells of a wild-type *Arabidopsis* leaf. (B) Enlarged chloroplasts in the mesophyll cells of an *msl2–1; msl3–1* mutant plant are indicated with asterisks. (C) MSL3-YFP is localized to the poles of dividing plastids in the hyptocotyl. Excitation was with 488 nm, chlorophyll signal (red) was collected with a 585 LP filter, and YFP signal (green) was collected with a 505–530 BP filter. Size bars are 5 μm.

becomes low (Pyke, 2006). Another possibility is that MSL2/3 affect the formation or stability of stromules, dynamic tubular extensions of the plastid envelope, commonly seen in the nongreen plastids of the leaf and root epidermis (Kohler and Hanson, 2000). In the *msl2–1; msl3–1* double mutant, most nongreen plastids are enlarged and spherical, and lack stromules, but occasionally plastids with long, tangled stromules are also observed. Alternatively, MSL2/3 may serve to release internal pressure that accumulates in the plastid as a result of metabolic activity or due to constriction during plastid division. Whether MSL2/3 interact directly with known components of the plastid division machinery is not known, though they do colocalize with the plastid division protein AtMinE (Haswell and Meyerowitz, 2006). Further investigation into the *msl2–1; msl3–1* mutant phenotype and into the nature of the mutant alleles should help distinguish between these possibilities.

V. OUTSTANDING QUESTIONS

A. How Have MscS-Like Proteins Evolved?

MscS family members have so far been found only in cell-walled eukaryotes. This is more likely a coincidence derived from the evolutionary history of the family than from the functional nature of the proteins, as some MscS-like proteins localize to organelles, which lack a wall. Furthermore, Class I proteins are only found in plants, while Class II proteins are found in plants, fungi, and a protist. A possible evolutionary scenario is one in which Class II proteins were present in the common ancestor of plants and fungi, and evolved to function at the plasma membrane (and perhaps the vacuolar membrane) of both lineages. Later, Class I proteins were introduced exclusively to the plant lineage by way of the cyanobacterial chloroplast ancestor, and a subset were then retargeted to the mitochondrial envelope. Such a transfer is thought to have occurred in the case of two mitochondrial ribosomal proteins (Adams *et al.*, 2002). In the animal lineage, the *MSL* gene family either lost the consensus sequence elements used in my analysis or was lost completely.

B. What Roles Do MS Ion Channels Play in Plant Biology?

This preliminary analysis of the MSL family only begins to answer the many questions about how MS ion channels may function in plants. If, like MscS, they are activated directly through changes in membrane tension, they might be expected to respond to stimuli that directly impact the plasma

membrane, like turgor pressure or temperature. These considerations predict that eukaryotic MscS-like proteins act as osmotic safety valves to prevent cellular rupture under extreme hypoosmotic shock. It is also possible that they mediate the perception of stimuli like touch. External mechanical stress is primarily borne by the wall of a plant cell, but could be transmitted to the plasma membrane by proteins that link the cell wall to the plasma membrane, such as the wall-associated kinases that have been identified in *Arabidopsis* (Gens *et al*., 2000; Anderson *et al*., 2001).

Other types of MS ion channels might also function in plant cells. The transient receptor potential (TRP) or degenerin/epithelial sodium channel (Deg/ENaC) families of MS ion channels are found in many animal cells and mediate the perception of pain, touch, and sound (Sukharev and Corey, 2004; Kung, 2005). Though none have yet been identified, it remains possible that homologues of these ion channel families may exist in plant genomes. Finally, molecules other than ion channels may act as mechanosensors in plant cells. For examples, there is immunological evidence for integrin-like activities in plant cells (Gens *et al*., 1996; Katembe *et al*., 1997; Faik *et al*., 1998; Laval *et al*., 1999; Nagpal and Quatrano, 1999).

C. Is Clustering of MS Ion Channels Important?

As described above, MSL2-GFP and MSL3-GFP fusion proteins form puncta on the plastid envelope, often at one or both poles of the organelle. This unusual intraplastidic localization may play a role specific to plastids, such as simply promoting equal distribution during plastid fission. However, there is evidence for clustering of other MS ion channels, such as MEC-4 and MEC-10, two mechanosensors involved in touch sensation in *Caenorhabditis elegans* (Zhang *et al*., 2004). Other MS ion channels open in bursts or show cooperative gating, consistent with functional physical interactions between individual channels (Szabo *et al*., 1990; Ding and Pickard, 1993a). Several of the MS ion channel activities in plant membranes listed in Table I are reported to localize in clusters on the membrane, or to activate in bursts. These data suggest that the formation of clusters is a common feature of MS ion channels, and may play a role in their function.

Clustering of MS ion channels may serve to amplify the force that is perceived or to amplify the signal that is produced. Pickard and colleagues propose that plant MS ion channels cluster at the vertex of a force-focusing network, composed of interactions between the plasma membrane, the cell wall, the cytoskeleton, and a specialized cortical ER (Gens *et al*., 2000; Pickard and Fujiki, 2005). The structural organization of this network would allow small global stresses to be locally magnified, thereby enhancing

sensitivity and spatially controlling the response. Alternatively, clustering might facilitate amplification of the signal output, as has been proposed for the methyl-accepting chemoreceptors of *E. coli* (reviewed in Parkinson *et al.*, 2005). In this case, cooperative interactions between dimers of different receptors is invoked to explain the observed signal gain.

VI. CONCLUSIONS

Molecular genetic, electrophysiological, and phylogenetic analyses are beginning to reveal how MscS-like proteins function in discrete cellular compartments, where they are implicated in the perception of membrane tension in *Arabidopsis* and *Chlamydomonas*. Building on this early work, a comprehensive analysis of the eukaryotic members of the *MSL* gene family should provide insight into several aspects of basic plant biology. Future studies may help reveal how plant and fungal cells use turgor control to respond to developmental and environmental cues. An understanding of plant mechanosensation may lead to improved crop productivity through the prevention of wind-dwarfing, dehydration, and salt sensitivity.

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