Arabidopsis MSL10 Has a Regulated Cell Death Signaling Activity That Is Separable from Its Mechanosensitive Ion Channel Activity

Kira M. Veley, Grigory Maksaev, Elizabeth M. Frick, Emma January, Sarah C. Kloepper, and Elizabeth S. Haswell

Department of Biology, Washington University, St. Louis, Missouri 63130

Members of the MscS superfamily of mechanosensitive ion channels function as osmotic safety valves, releasing osmolytes under increased membrane tension. MscS homologs exhibit diverse topology and domain structure, and it has been proposed that the more complex members of the family might have novel regulatory mechanisms or molecular functions. Here, we present a study of MscS-Like (MSL10) from Arabidopsis thaliana that supports these ideas. High-level expression of MSL10-GFP in Arabidopsis induced small stature, hydrogen peroxide accumulation, ectopic cell death, and reactive oxygen species- and cell death-associated gene expression. Phosphomimetic mutations in the MSL10 N-terminal domain prevented these phenotypes. The phosphorylation state of MSL10 also regulated its ability to induce cell death when transiently expressed in Nicotiana benthamiana leaves but did not affect subcellular localization, assembly, or channel behavior. Finally, the N-terminal domain of MSL10 was sufficient to induce cell death in tobacco, independent of phosphorylation state. We conclude that the plant-specific N-terminal domain of MSL10 is capable of inducing cell death, this activity is regulated by phosphorylation, and MSL10 has two separable activities—one as an ion channel and one as an inducer of cell death. These findings further our understanding of the evolution and significance of mechanosensitive ion channels.

INTRODUCTION

How individual cells sense and respond to environmental stresses and how they do so in the context of a multicellular organism remain important biological problems. Much is unknown about the perception of stimuli that are mechanical in nature, such as touch, gravity, and membrane stretch (i.e., mechanotransduction), though it is clear that these types of signals are important regulators of growth and development in bacteria, plants, and animals (Nakayama et al., 2012; Steffens et al., 2012; Lai et al., 2013; Mousavi et al., 2013; Yan et al., 2013). A particularly well-studied molecular mechanism for the perception and transduction of mechanical signals is provided by mechanosensitive (MS) ion channels, channels that open directly or indirectly in response to membrane tension (Amadotti and Challie, 2010; Kung et al., 2010; Sukharev and Sachs, 2012). Genes that are predicted to encode MS channels are found in all three domains of life, in a number of evolutionarily unrelated families (Liu et al., 2010; Nakayama et al., 2012; Sukharev and Sachs, 2012; Prole and Taylor, 2013).

The bacterial mechanosensitive channel of small conductance (MscS) from Escherichia coli is a leading model for the study of mechanosensation (Haswell et al., 2011; Martinac, 2011; Naismith and Booth, 2012). The four existing crystal structures of bacterial MscS (Bass et al., 2002; Steinbacher et al., 2007; Wang et al., 2008; Lai et al., 2013) reveal a channel comprising seven identical subunits. Each subunit contains three transmembrane (TM) helices, with the third TM helix of each monomer lining the pore. This pore extends into the vestibule of a large cytoplasmic chamber that may serve to influence the composition of ions that pass through the channel (Gaminí et al., 2011; Zhang et al., 2012; Cox et al., 2013). Along with several other MS ion channels in the bacterial membrane, MscS facilitates survival of hypo-osmotic shock by releasing osmolytes when membrane tension increases beyond a certain threshold and is frequently referred to as an “osmotic safety valve” (Blount and Moe, 1998; Leiva et al., 1999; Sotomayor et al., 2006; Boer et al., 2011; Reuter et al., 2014).

Homologs of MscS are found in nearly all bacterial species (Pivetti et al., 2003; Lai et al., 2013; Martinac et al., 2013), protozoa (Prole and Taylor, 2013), archaea (Palmieri et al., 2009), some fungi (Nakayama et al., 2012), and all plant genomes so far analyzed (Wilson et al., 2013) but have not been identified in animals. The region of sequence similarity between MscS and other members of the MscS superfamily is restricted to a relatively small portion of the protein that includes the pore-lining helix of MscS and ~100 amino acids of the upper cytoplasmic domain (Kloza and Martinac, 2002; Pivetti et al., 2003; Balleza and Gómez-Lagunas, 2009; Haswell et al., 2011). E. coli MscS is among the smallest members of its eponymous family of proteins, and deletion studies indicate that it contains little nonessential protein sequence (Miller et al., 2003b; Schumann et al., 2004). However, other MscS family members show substantial variation in size and topology, containing anywhere from 3 to 12 TM helices (Haswell et al., 2011) and a variety of domains not found in MscS, such as large cytoplasmic loops, N- or C-terminal extensions, extracellular domains, and ion or cyclic nucleotide binding motifs (Li et al.,...
overexpression of the MSL10 cDNA from the constitutive and strong cauliflower mosaic virus 35S promoter (P35S) (Odell et al., 1985; Jefferson et al., 1987). Green fluorescent protein (GFP) was fused to the C terminus of MSL10; this tag has no effect on the channel properties of MSL10 when expressed in Xenopus oocytes (Maksaev and Haswell, 2012) or root protoplasts (Haswell et al., 2008). We observed that MSL10-GFP overexpression lines exhibited a range of phenotypes, including dwarfism and brown patches on the leaf margins (Figure 1A, top row). Similar phenotypes were observed in plants grown under short-day conditions (8 h of light; Supplemental Figure 1A), indicating that they are not the result of a stressful light regime. We selected three T2 lines with a range of phenotypic severity for further analysis. Elevated MSL10-GFP expression in these lines was confirmed by RT-PCR (Figure 1B, top panel).

The brown patches on the leaves of plants overexpressing MSL10-GFP resembled lesions resulting from the activation of programmed cell death (PCD), a highly regulated process that is initiated during senescence or immune response (Gar and Aamasino, 1997; Diaz et al., 2006; Kothoni and Gachomo, 2006; Gill and Tuteja, 2010). One hallmark of PCD is the production of reactive oxygen species (ROS) like H$_2$O$_2$, which are used as both a signal for and a facilitator of stress and PCD-associated pathways (Desikan et al., 1998; Mittler et al., 2004; Cui et al., 2013). We therefore stained leaf number 4 or 5 from 2-week-old wild-type plants and MSL10-GFP overexpression lines with 3,3'-diaminobenzidine (DAB) or 3-week-old plants with trypan blue (Figure 1A, middle and bottom rows). We observed that all three overexpression lines stained with DAB had increased levels of a dark brown precipitate compared with the wild type, indicative of the increased presence of H$_2$O$_2$. Additionally, the two lines that appeared to have the highest levels of H$_2$O$_2$ production, 12-3 and 15-2, also stained with trypan blue, a commonly used indicator of lesion development, at 3 weeks of age. Trypan blue staining was not detected in leaves from the wild type or line 7-1 (Figure 1A). The observed levels of H$_2$O$_2$ accumulation and lesion formation roughly correspond with the amount of MSL10 overexpression; line 12-3 was the most severe and line 7-1 had a milder phenotype (Figures 1A and 1B). Thus, overexpression of MSL10-GFP resulted in the accumulation of H$_2$O$_2$ in leaf cells and patches of ectopic cell death.

After 3 weeks of growth, overexpression lines had acquired significantly less mass (milligrams per rosette) than the wild type, weighing 4 to 10 times less than the untransformed wild-type control (Figure 1C). The average leaf epidermal pavement cell area in each line was calculated from confocal laser scanning microscopy (CLSM) images of the adaxial surfaces of rosette leaves stained with propidium iodide (PI) to highlight the plasma membrane. Examples of the images used for this analysis are shown in Figure 1D. We found that the average area of an adaxial pavement cell in the overexpression lines was less than half that of the wild type (Figure 1D). Thus, the MSL10-GFP overexpression lines may be smaller due to reduced cell expansion.

To determine which cell death pathways might be induced in response to MSL10-GFP overexpression, we compared the expression levels of genes involved in a range of cell death- and ROS-associated pathways between wild-type and MSL10-GFP overexpressing plants. We selected for analysis five genes reported to be induced by either biotic or abiotic stress and involved in ROS production, PCD, or senescence. SAG12 encodes

RESULTS

Overexpression of MSL10-GFP Results in Small Stature, H$_2$O$_2$ Accumulation, Ectopic Cell Death, and the Transcriptional Activation of Reactive Oxygen Species- and Cell Death-Associated Genes

We generated stably transformed Arabidopsis homozygous lines in the Columbia-0 ecotype (Col-0; referred to here as the wild type) overexpressing the MSL10 cDNA from the constitutive and strong
Figure 1. Phenotypes Associated with the Overexpression of MSL10-GFP in Arabidopsis.

(A) Wild-type (Col-0) plants, three independent homozygous transgenic T2 lines (also in the Col-0 background) expressing MSL10-GFP, and two independent homozygous T2 lines expressing MSL10^{4D}-GFP from the cauliflower mosaic virus 35S promoter (P35S) are compared. The numbers above images designate the names of the specific transgenic lines. Top row: images of 3-week-old plants grown at 21°C under a 24-h light regime. Bar = 0.5 cm. Middle row: bright-field images of leaf 4 or 5 from 2-week-old plants from the indicated lines stained with DAB for the presence of hydrogen peroxide. Bar = 0.1 cm. Bottom row: bright-field images of leaf 4 or 5 from 2-week-old plants from each line stained with trypan blue to assess lesion formation. Bar = 200 μm.

(B) RT-PCR analysis of selected genes in wild-type and MSL10-GFP overexpression lines. A single technical replicate is shown for each line. cDNA was synthesized from RNA extracted from rosette tissue from 3-week-old plants. At least 10 individuals were pooled for each sample. ACTIN expression was used as a control.

(C) Average fresh weight (mg) of rosettes from 10 17-d-old soil-grown plants. Error bars = sd. *P value < 0.01 relative to Col-0 (Student’s t test).

(D) Quantitative analysis of lesion area in wild-type and MSL10-GFP overexpression lines. WT, wild-type; P35S:MSL10-GFP, transgenic line expressing MSL10-GFP from the 35S promoter; P35S:MSL10^{4D}-GFP, transgenic line expressing MSL10^{4D}-GFP from the 35S promoter. error bars = SD. *P value < 0.01 relative to Col-0 (Student’s t test).

(E) Western blot analysis of MSL10-GFP and MSL10^{4D}-GFP expression levels in wild-type and transgenic lines. MSL10-GFP, MSL10^{4D}-GFP, α-GFP, and α-tubulin were detected using specific antibodies.
a cysteine protease and is a well-established transcriptional marker of senescence (Gan and Amasino, 1997; Fischer-Kilbienski et al., 2010; Brusslan et al., 2012; Koyama et al., 2013), OSM34 encodes an osmotin-like protein (Capelli et al., 1997; Abdin et al., 2011; Sharma et al., 2013), and DOX1 encodes an α-dioxygenase that protects plant cells from oxidative stress and plays an important role in mediating pathogen-induced cell death (De León et al., 2002; Blanco et al., 2005). PERX34 encodes a peroxidase that promotes H₂O₂ production in response to biotic stresses (Bindschedler et al., 2006; Daudi et al., 2012), and KTI1 encodes a trypsin inhibitor that modulates PCD (Li et al., 2008). RT-PCR was performed on cDNA synthesized from RNA extracted from 3-week-old whole rosette tissue. As expected, all five genes were expressed at low to undetectable levels in healthy wild-type plants. However, all of the genes tested were the most highly expressed in line 12-3, which also had the highest level of MSL10-GFP transcript (Figure 1B) and MSL10 (Figure 1E) production, the darkest DAB staining, and the most striking lesion formation (Figure 1A). While the 7-1 line showed intermediate levels of MSL10 transcript and an intermediate reduction in rosette size, it exhibited low DAB and trypan blue staining, and SAG12, OSM34, and DOX1 transcripts were not detected (Figure 1). These observations are consistent with recent evidence that many measures of abiotic stress do not respond linearly to stress and that shoot growth is often the most sensitive parameter (Claeys et al., 2014). These data indicate that a number of biotic and abiotic stress pathways are interesting at the transcriptional level by high levels of MSL10-GFP. Taken together, the data presented in Figure 1 show that ectopic overexpression of MSL10 promotes cell death and ROS accumulation in stably transformed Arabidopsis lines.

The MSL10 N-Terminal Domain Is Phosphorylated in Vivo and Is Specific to Plants

At least nine independent high-throughput proteomic analyses of phosphorylated proteins have identified phosphorylated residues in peptides derived from the N-terminal region of MSL10, which is predicted to be soluble and cytoplasmic (amino acids 1 to 164; Figure 2). MSL10 S29, S46, S48, S57, S128, S131, and T136 are in vivo phosphorylated in suspension cells (Nühse et al., 2004; Benschop et al., 2007; Sugiyama et al., 2008; Li et al., 2009; Nakagami et al., 2010), seedlings (Engelsberger and Schulze, 2012; Wang et al., 2013), and adult plants (Reiland et al., 2009, 2011). These data are summarized in Figure 2 and in Supplemental Table 1 and can also be accessed via the PhosPhAt 4.0 database (http://phosphat.mpimp-golm.mpg.de). The soluble N-terminal domain of MSL10 is specific to MSL10 and its orthologs in other plants (Supplemental Figure 2) and does not share homology with any region in MascS (Supplemental Figure 3). Indeed, this region does not contain any identified conserved functional domains and shows the least sequence homology among the Class II MSLs from Arabidopsis (49% amino acid sequence identity, compared with 68% in the rest of the protein). We therefore sought to determine if this MSL10-specific sequence plays an important functional role and if such a function might be regulated by phosphorylation.

Phosphomimetic Mutations in the MSL10 N-Terminal Domain Prevent the Phenotypes Caused by Overexpression of Wild-Type MSL10-GFP

To investigate potential regulatory roles for the phosphorylation state of the MSL10 N-terminal domain, we made a series of point mutations at the four residues identified as phosphorylated under the greatest range of conditions (S57, S128, S131, and T136; Supplemental Table 1). We introduced genetic lesions that mimicked either the phosphorylated or the unphosphorylated state, a well-established technique used to study the functional relevance of potential phosphorylation sites (Kaufman et al., 1989; Cui et al., 2004; Qiao et al., 2012). These four residues were changed to either aspartate or glutamate to mimic constitutive phosphorylation depending on which was most convenient given the wild-type mRNA sequence (MSL10 S57D, S128D, S131E, and T136D are designated MSL104D here for simplicity) or to alanine to prevent phosphorylation (designated MSL104A). Overexpression of MSL104D-GFP caused none of the phenotypes observed in the wild-type MSL10-GFP overexpression lines, including H₂O₂ production (Figure 1A, middle row), cell death (Figure 1A, bottom row), reduced plant size (Figures 1A, top row, and 1C), or reduced cell expansion (Figure 1D), despite having high levels of MSL10-GFP mRNA (Figure 1B) and MSL10 (Figure 1E). Overexpression of MSL104D-GFP did not activate SAG12, OSM34, DOX1, or PERX34 and resulted in only slightly increased tran$scripti$onal activation of KTI1 (Figure 1B). PERX34 levels were also elevated in 5-d-old seedlings overexpressing wild-type MSL10-GFP compared with the wild type, but not in seedlings overexpressing MSL104D-GFP. SAG12, OSM34, DOX1, and KTI1 did not exhibit this pattern in seedlings (Supplemental Figure 1B). We were unable to isolate plants expressing detectable levels of MSL104A-GFP (~200 T1 individuals were analyzed by CLSM), which will be discussed further below. These data indicate that the identities of the residues S57, S128, S131, and T136 were critical for the observed MSL10 overexpression phenotypes and that the phosphorylation status of these residues may be a vital component of MSL10 function.

Figure 1. (continued).

(D) Top: representative CLSM images of cell outlines analyzed by ImageJ. Samples were taken from the distal quadrant of the 4th newest leaf from 3-week-old plants, and cells from the adaxial surface were imaged. Bar = 50 μm. Bottom: average area of n > 100 cells per background. Error bars = SD. *P value < 0.01 relative to the wild type (Student’s t test).

(E) Immunoblot of extracts from the stably transformed Arabidopsis lines shown in (A). Blot was detected with an anti-GFP primary antibody (top), then stripped and reprobed with anti-α-tubulin primary antibody (bottom). Protein sizes are indicated at the right according to a commercially available standard.
The Phosphorylation State of the MSL10 N-Terminal Domain Regulates Its Ability to Induce Cell Death in a Transient Expression System

Because we were unable to isolate stably transformed Arabidopsis overexpressing MSL10A-GFP, and to provide further support for MSL10 as an inducer of cell death, we employed a transient expression system to more systematically investigate the effect of phosphorylation on MSL10 function and to quantitatively assess cell death. We used transient overexpression of MSL10 variants in tobacco (Nicotiana benthamiana) leaves to analyze the effect that the number of phosphorylated residues had on MSL10 activity by expressing MSL10 with two (S128 and S131), four (S57, S128, S131, and T136), or seven (S29, S46, S48, S57, S128, S131, and T136) phosphorylated residues mutated. Wild-type (P3SS:MSL10-GFP), phosphomimetic (P3SS:MSL10-GFP), or alanine substitution (P3SS:MSL10A-GFP) versions of MSL10 were transiently overexpressed in tobacco leaves via infiltration with Agrobacterium tumefaciens transformed with the binary vector pK7FWG2 (Karimi et al., 2002) harboring the appropriate MSL10 sequences. A plasmid encoding the p19 gene of tomato bushy stunt virus, which is often used in transient expression systems to inhibit transgene silencing (Voinnet et al., 2003; Waadt and Kudla, 2008; Garabagi et al., 2012), was coinfiltrated with each construct. Three days after infiltration, successful expression was confirmed through GFP fluorescence (Figure 3A, top row). By 5 d postinfiltration, GFP signal had disappeared in some cells of the leaves expressing P3SS:MSL10-GFP and P3SS:MSL10A-GFP (Figure 3A, second row), and trypan blue staining indicated the appearance of patches of dead cells in leaves expressing these constructs (Figure 3A, third row). To quantify this effect at the cellular level, the percentage (averaged over three different infiltration experiments) of dead epidermal pavement cells from the abaxial surface of leaves expressing MSL10-GFP variants was determined by PI and fluorescein diacetate (FDA) viability staining (Chaves et al., 2002). Vacuolar integrity was also used as an indication of vitality and was assessed using a combination of false differential interference contrast (pseudocolored gray) and localization of FDA. Representative images of cells classified as dead or alive using this method are presented in Figure 3A, bottom row, and additional examples of raw data used in the analysis, along with further information about the assay, can be found in Supplemental Figure 4. Statistical differences between these samples were analyzed by one-way ANOVA and Tukey’s test, and the “a” and “b” classes indicated in Figure 3B represent significant differences with P < 0.05.

Consistent with what we observed in stably transformed Arabidopsis lines, we found that expression of wild-type MSL10-GFP resulted in the death of ∼25% of tobacco epidermal pavement cells, while expression of p19 alone resulted in the death of just 5% of the cells 5 d after infiltration (Figure 3B). Furthermore, replacing the residues known to be phosphorylated in vivo with phosphomimetic amino acids completely ameliorated MSL10-induced cell death. Infiltration with MSL10D-GFP and MSL10A-GFP resulted in levels of cell death that were statistically inseparable from that observed with the p19 only control, causing ~9 and 7% cell death, respectively (Figure 3A, right panel; Figure 3B, group “a”). Additionally, expressing MSL10D-GFP caused 14% of cells to die, a value which could not be statistically separated from either group “a” or “b” (2; Figure 3B), suggesting a threshold effect with respect to the number of residues altered to mimic phosphorylation.

On the other hand, replacing two, four, or all seven of the in vivo phosphorylated residues with alanine resulted in increased levels of cell death, with 20, 29, and 35% cell death observed, respectively. These samples were statistically inseparable from each other and from cells infiltrated with the wild-type MSL10-GFP (Figure 3B, group “b”; Figure 3A, left panel). All MSL10 variants tested produced similar GFP signal and transformation efficiency at 3 d postinfiltration (Figure 3A, top row) and similar protein levels in whole tobacco leaf extracts at 5 d postinfiltration (Figure 3C). Taken together, these data demonstrate that the phosphorylation state of the MSL10 N-terminal domain regulates its ability to promote cell death when overexpressed in plant cells. A likely explanation for the observation that ectopic overexpression of wild-type MSL10-GFP resulted in high levels of cell death in either stably transformed Arabidopsis (Figure 1) or transiently expressing tobacco epidermal cells (Figure 3) is that the cell cannot maintain all copies of MSL10 in the phosphorylated state when it is expressed at high levels and that the resulting subpopulation of unphosphorylated MSL10 is capable of inducing cell death. The phosphorylated form of MSL10, the inactive form of the protein with respect to inducing cell death, is thus likely to be the default state of MSL10 in vivo, consistent with the fact that at least nine phosphoproteomic studies on a variety of tissue types under a multitude of conditions
all identify the MSL10 N-terminal domain as phosphorylated (Supplemental Table 1).

Preventing or Mimicking Phosphorylation of the MSL10 N-Terminal Domain Does Not Demonstrably Affect Subcellular Localization, Assembly, or Electrophysiological Properties of the Channel

To better understand the molecular basis for these results, we tested the effects of the same point mutations on three characteristics of the MSL10 protein: subcellular localization, interaction between monomers, and electrophysiological properties. It is well established that the phosphorylation state of ion channels can affect their trafficking and localization (Maurel et al., 2009; Bayle et al., 2012), so we tested whether phosphorylation altered the subcellular localization of MSL10-GFP variants when transiently overexpressed in tobacco. We transiently co-expressed MSL10 variants and organelle-specific markers fused to mCherry (Nelson et al., 2007) and looked for colocalization using CLSM. As previously shown with stably transformed Arabidopsis expressing MSL10-GFP under the control of its own promoter (Haswell et al., 2008), wild-type MSL10-GFP exhibited localization to both the ER and the PM (Figure 4A, second column). The left panel is a representative example of an image showing cells scored as “dead” due to PI staining in the nucleus and lack of FDA or GFP signal, while all cells shown in the right panel were scored as “live.” Bar = 10 µm. (B) Percentage of dead cells in tissue expressing MSL10-GFP variants coinfiltred with p19. At least 120 cells were counted from each construct from two separate infiltration experiments, each consisting of three infiltrated leaves. Statistical differences were analyzed by one-way ANOVA and Tukey’s test, and groups that did not differ significantly are indicated by the same letter (a and b, P < 0.05). p19 alone was used as a background control. (C) Immunoblot analysis of MSL10-GFP from samples used in one of the tobacco expression experiments in (B). Protein extracts from leaves infiltrated with p19 alone were used as a negative control. Top: anti-GFP antibody. Bottom: anti-α-tubulin loading control. The migration of bands from a commercially available protein size standard is indicated at the right.

Figure 3. Quantification of Cell Death in Tobacco Cells Transiently Expressing MSL10-GFP Variants.

(A) Images of the abaxial surface of tobacco leaves after infiltration with Agrobacterium harboring the indicated MSL10-GFP variant and the expression enhancer p19. Top row: CLSM image of GFP expression (pseudocolored green) in epidermal cells 3 d postinfiltration. Bar = 50 µm. Second row: GFP expression 5 d postinfiltration. Bar = 50 µm. Third row: bright-field images of tobacco leaf samples 5 d postinfiltration stained with trypan blue to assess lesion formation. Bar = 500 µm. Bottom row: representative images of live and dead pavement cells in tobacco leaves 5 d postinfiltration. Leaves were stained with FDA (pseudocolored yellow) and PI (pseudocolored red). The left panel is a representative example of an image showing cells scored as “dead” due to PI staining in the nucleus and lack of FDA or GFP signal, while all cells shown in the right panel were scored as “live.” Bar = 10 µm.
Figure 4. Effect of Phosphomimetic and Alanine Substitution Mutations on MSL10 Behavior.

(A) Subcellular localization in epidermal cells from tobacco transiently expressing the same GFP constructs stably transformed into Arabidopsis. CLSM images were taken 3 d postinfiltration, before high levels of cell death were seen. To assess subcellular localization, MSL10 variants were coinfiltrated with either an ER (ER-mCherry) or a plasma membrane (PM-mCherry) marker (Nelson et al., 2007). MSL10-GFP was pseudocolored green, and the ER marker (top row) or the PM marker (middle row) was pseudocolored magenta (see online version for color); colocalization appears white. Bottom row: MSL10-GFP-expressing cells were plasmolyzed for 5 min in 1 M NaCl. Arrowheads indicate GFP signal in Hechtian strands. Bars = 10 μm.

(B) Split-ubiquitin yeast two-hybrid analysis of protein-protein interactions among wild-type and mutant forms of MSL10. Each variant of MSL10 was fused to either Cub (rows) or Nub (columns), and haploid yeast containing a single construct were mated. Growth of diploid cells after 5 d on minimal media indicated an interaction. The N-terminal half of ubiquitin alone (NubG) was used as a negative control. The "G" in NubG indicates a mutation within Nub that decreases the incidence of false positive interactions. Wild-type Nub, which contains no such mutation and is thus more promiscuous when used as a binding partner, was used as a positive control.

(C) Top: CLSM images of the edge of X. laevis oocytes expressing MSL10-GFP (pseudocolored green) 7 d after injection with cRNA. Mock injections with water were used as a control. Bars = 100 μm. Bottom: representative traces of tension-induced currents in inside-out excised patches from X. laevis oocytes expressing MSL10 variants. Records were made from the patches in pipettes with BN –5 in symmetric ND96 buffer. Membrane potentials were clamped at –40 (wild type) or –30 mV (MSL10^A and MSL10^D) during the application of a symmetric triangular 5-s pressure ramp to –70 (wild type and MSL10^A) or –60 mm Hg (MSL10^D). Note that hysteresis (wherein the last channel closes at lower tensions than the first channel opens) was observed in all traces. A quantification of opening threshold tension and open/close threshold tension ratios is shown in Supplemental Figure 5.

(D) Current/voltage curves of MSL10-GFP (open circles), MSL10^A-YFP (diamonds), and MSL10^D-YFP (closed circles) under membrane tension (n = 3 oocytes for each protein). The GFP tag does not alter MSL10 conductance (Maksaev and Haswell, 2012).

[See online article for color version of this figure.]
could interact with MSL107A or MSL107D in a yeast growth assay

between membrane-localized proteins (Ludewig et al., 2003; hybrid assay, which is specifically designed to detect interactions between membrane-localized proteins (Ludewig et al., 2003; Ebert et al., 2013).

differ between the wild-type and phospho-mutant forms. We tested for interactions among MSL10 variants using the mating-based split-ubiquitin yeast two-hybrid assay, which is specifically designed to detect interactions between membrane-localized proteins (Ludewig et al., 2003; Obrdlik et al., 2003; Koprowski et al., 2011). As expected, we found that wild-type MSL10 was capable of self-interaction and also observed that MSL10 could interact with MSL107A or MSL107D in a yeast growth assay (Figure 4B, top row and left column). Furthermore, MSL107A and MSL107D mutant forms were able to interact efficiently with themselves (Figure 4B, second and third rows and columns). None of the variants tested showed evidence of autoactivating the reporter (Figure 4B, empty NubG column). These data indicate that MSL10 monomer-monomer interactions in the yeast two-hybrid assay were unaffected by either type of lesion at phosphorylated residues.

A third potential role for phosphorylation of the MSL10 N-terminal domain is in the regulation of its ion channel properties, as has been shown for TPK1 (Latz et al., 2007), TRP (Voolstra et al., 2010), CLH-3b (Yamada et al., 2013), and PIP2 (Prado et al., 2013). Mutations outside the membrane-spanning pore of bacterial MscS homologs alter channel conductance (Zhang et al., 2013). Mutations outside the membrane-spanning pore of bacterial MscS homologs alter channel conductance (Zhang et al., 2013). Gating and conductance properties of MSL10. All MSL10 variants, and alanine substitution mutations on the mechanosensitive domain is in the regulation of its ion channel properties, as has been shown for TPK1 (Latz et al., 2007), TRP (Voolstra et al., 2010), CLH-3b (Yamada et al., 2013), and PIP2 (Prado et al., 2013). Mutations outside the membrane-spanning pore of bacterial MscS homologs alter channel conductance (Zhang et al., 2013). Gating and conductance properties of MSL10. All MSL10 variants, and alanine substitution mutations on the mechanosensitive domain (Supplemental Figure 5). Furthermore, we did not observe any differences in tendency to flicker or in the average number of active channels in each patch between the variants. Finally, though the variability in these measurements was high (the membrane tension in a membrane patch produced by a given amount of pressure can vary widely due to differences in patch size and geometry; Suchyna et al., 2009), all three MSL10 variants had similar threshold tensions for opening and similar ratios of opening to closing tensions (Supplemental Figure 5). Current/voltage (I/V) curves measured for single-channel openings between −50 and +50 mV were identical for MSL10-GFP, MSL10A-YFP, MSL10A-YFP, MSL10D-YFP, and MSL10D-YFP (Figures 4D; Supplemental Figure 5). Taken together, the data shown in Figure 4 establish that the phosphorylation state of the MSL10 N-terminal domain does not appreciably govern the subcellular localization, oligomerization, or channel properties of MSL10.

The MscH Homology Domain of MSL10 Is Not Required for Its Phosphorylation-Dependent Induction of Cell Death

The region of homology between the bacterial channel MscS and MSL10 is relatively small and comprises the most C-terminal TM helix of each protein and subsequent 100 amino acids (Figure 2). In MscS, these sequences form the channel pore and the upper vestibule of the cytoplasmic domain. The corresponding portion of MSL10 is therefore likely to be essential for its function as a MS channel. To test this, we expressed in X. laevis oocytes a version of MSL10 that included the N-terminal cytoplasmic domain and the first four TM helices (MSL101-316; Figure 5A) fused to YFP. MSL101-316-YFP was expressed well and localized to the plasma membrane, but no mechanically activated channel activity was detected (17 patches pulled from six oocytes), even at membrane tensions that were close to lytic (Figure 5B). To determine if the MscH homology domain of MSL10 was required to induce cell death, we transiently overexpressed MSL10A1-316 and MSL10D1-316 fused to GFP in tobacco leaves. Truncation of MSL10 at amino acid 316 did not appreciably alter its expression level or its localization (Figure 5C). Furthermore, as shown in Figure 5D, transient overexpression of MSL10A1-316-GFP (group c) caused even more cell death than the full-length channel MSL10A-GFP (group a), while the cell death observed in leaf cells overexpressing MSL10D1-316-GFP was statistically indistinguishable from that induced by expression of full-length MSL10D-GFP or p19 alone (group b). We conclude that the ability of MSL10 to promote cell death in a phosphorylation state-specific manner is genetically and physically separable from its MscH homology domain and from its activity as a mechanosensitive ion channel, a conclusion that is further supported below.

The Cytoplasmic N-Terminal Domain of MSL10 Is Sufficient to Induce Cell Death in Tobacco

To further delineate the portion of MSL10 responsible for the production of phosphorylation-dependent cell death, we performed an analysis similar to that shown in Figure 5, but with only the soluble N-terminal domain of MSL10 (amino acids 1 to 164; Figure 6A) fused to GFP (MSL101-164-GFP). As expected, transient overexpression of the full-length channel MSL10A-GFP caused a large percentage (~50%) of the cells to die (group a; Figure 6C), while expression of full-length MSL10D-GFP produced the same amount of cell death as p19 alone (group b). To our surprise, MSL101-164-GFP was capable of inducing cell death to the same degree as the full-length MSL10A-GFP protein (group a; Figure
6C). Furthermore, all three variants tested (MSL10\textsubscript{1-164}-GFP, MSL10\textsubscript{1-164}-GFP, and MSL10\textsubscript{1-164}-GFP) caused the same level of cell death (group a; Figure 6C). Thus, the soluble N-terminal domain of MSL10 is capable of inducing cell death to the same degree as the full-length protein when overexpressed in plant cells but is no longer governed by phosphorylation state. All three variants of the soluble N-terminal domain localized to the cytoplasm and nucleus (the latter indicated by 4',6-diamidino-2-phenylindole [DAPI] signal; Figure 6B, bottom row). Full-length MSL10-GFP variants localized to the cell periphery.

Figure 5. Mechanosensitive Ion Channel Activity of MSL10\textsubscript{1-316}-GFP and Its Effects on Cell Death and Subcellular Localization.

(A) Cartoon of the MSL10\textsubscript{1-316} monomer. The cytoplasmic N-terminal domain is dark red and the phosphorylated residues within it are black. Most of the cytoplasmic loop, the 5th and the 6th TM helices, and the cytoplasmic C terminus are deleted, as indicated by dashed circles. These deleted portions include the MscS homology domain (blue dashed circles), which forms the channel pore in \textit{E. coli} MscS.

(B) Top: confocal scan of the periphery of an oocyte expressing MSL10\textsubscript{1-316}-YFP showing strong fluorescent signal. Image was taken 7 d after injection of RNA. Bar = 100 \textmu m. Bottom: a representative trace showing an absence of mechanosensitive activity even at high transmembrane pressures. The membrane potential was clamped at $-50$ mV.

(C) CLSM images of abaxial tobacco leaf epidermis from plants transiently expressing variants of full-length MSL10-GFP (top row) or MSL10\textsubscript{1-316}-GFP (bottom row). All seven phosphorylated residues were mutated as indicated above each image. GFP signal is pseudocolored green. Images were taken 3 d postinfiltration. Bar = 50 \textmu m.

(D) Percentage of dead cells in tissue expressing the same constructs as in (C) 5 d postinfiltration. The number and type of mutated residues are indicated below each bar, and the \textit{p19} plasmid alone was used as a negative control. Cells were counted over two independent infiltration experiments, each consisting of three infiltrated leaves per construct, \( n > 100 \). Statistical differences were analyzed by one-way ANOVA and Tukey’s test, and groups that differed significantly are indicated by different letters (a, b, and c, \( P \) value < 0.05). [See online article for color version of this figure.]
as previously observed (Figure 6B, top row). These data show that the N-terminal domain of MSL10 has cell death-promoting activity that does not require the pore-forming portion of the channel or even tethering to the plasma membrane. Furthermore, physical separation from the rest of the channel relieved the dependence of this activity on dephosphorylation. The in silico simulations of protein folding presented in Supplemental Figure 6 are consistent with these conclusions, as they show the cytoplasmic N-terminal domain forming a stable structure and no gross structural rearrangements associated with different phosphorylation states.

**DISCUSSION**

While little is known about members of the MscS family in multicellular eukaryotes, we previously speculated that the diversity of domains and topological complexity present in the family may allow for unique functions and regulatory mechanisms to evolve, each attuned to the needs of the organism in question. Furthermore, we suggested that such unique functions might not be restricted to the release of osmolytes (Haswell et al., 2011; Wilson et al., 2013). The data presented here provide support for these ideas, employing as a test case the MscS-Like MS channel MSL10 from Arabidopsis. We show that MSL10 is capable of acting as both a MS ion channel and as a promoter of ROS accumulation and cell death. We further show that the latter activity can be attributed to the soluble N-terminal domain of MSL10 and that it is negatively regulated by phosphorylation of several serine and threonine residues. These results pose a new set of questions; below, we discuss some of these questions and describe a working model that explains our observations and provides a platform from which to begin future investigations.

**MSL10 as a Sensor of Membrane Tension with Multiple Outputs**

Here, we show that a cell death-associated function of MSL10 is tied to a plant-specific region of the protein and is regulated by phosphorylation. As the cell death-inducing activity of MSL10 domain (blue dashed circles) and all transmembrane helices (gray dashed circles).

**Figure 6.** Effects of Expressing the Soluble N-Terminal Domain of MSL10 (MSL10_{1-164}-GFP) on Cell Death and Subcellular Localization. 

(A) Cartoon of MSL10_{1-164}. The cytoplasmic N-terminal domain is dark red (see online version for color) and the phosphorylated residues within it are black. All six TM helices, the cytoplasmic loop between TM4 and TM5, and the C-terminal domain are deleted, as indicated by dashed circles. These deleted portions include the MscS homology domain (blue dashed circles) and all transmembrane helices (gray dashed circles).

(B) CLSM images of the abaxial tobacco leaf epidermis from plants transiently expressing either full-length MSL10-GFP (top) or MSL10_{1-164}-GFP, (bottom), 3 d postinfiltration. The phosphovariant of MSL10 used in the infiltration is indicated above each image. GFP signal is pseudocolored green and the DNA marker DAPI pseudocolored blue. Colocalization of GFP and DAPI signals appears aqua. Bar = 10 \( \mu \)m.

(C) Percentage of dead cells in tissue expressing the same constructs as in (B) 5 d postinfiltration. The number and type of mutated residues are indicated below each bar, and the \( p19 \) plasmid alone was used as a negative control. Cells were counted over two separate infiltration experiments, each consisting of three infiltrated leaves per construct. \( n > 100 \). Statistical differences were analyzed by one-way ANOVA and Tukey’s test, and groups that do not differ significantly are indicated by the same letter (a and b, \( P \) value < 0.05). \( p19 \) alone was used as a negative control. 

[See online article for color version of this figure.]
remains intact even after the removal of its mechanosensitive capabilities, MSL10 can be considered to have at least two genetically separable outputs—ion flux and triggering cell death. However, it is reasonable to suppose that there is a functional link between the two. Our working model for MSL10 function is presented in Figure 7. Like *E. coli* MscS, MSL10 is capable of releasing osmolytes immediately in response to membrane tension. However, unlike MscS, MSL10 is also capable of inducing cell death, and we hypothesize that this activity is also normally activated in response to membrane tension. These two responses could provide both a short- and a more long-term response to membrane deformation. In a multicellular eukaryote like *Arabidopsis*, the subset of cells most affected by mechanical stress might be sacrificed for the greater good of the entire organism, as in the initiation of localized programmed cell death during the hypersensitive response to pathogenic invasion (Coll et al., 2011; Senthil-Kumar and Mysore, 2013). This idea is supported by the observation that, while hypo-osmotic shock causes cell death in bacteria due to lysis (Levina et al., 1999), eukaryotic cells respond to hypo-osmotic shock by undergoing a regulated process of cell death (Okada et al., 2001; Nakayama et al., 2012). Figure 7 illustrates a model wherein the structural rearrangements associated with increased membrane tension lead to both opening of the channel pore (and thereby to ion flux across the membrane) and to exposure of the N-terminal domain to a phosphatase (and thereby to its cell death promoting activity). However, our data do not rule out the possibility that the ion channel activity of MSL10 is indirectly involved in the dephosphorylation of the soluble N-terminal domain. Below, we propose and discuss a few questions we aim to address in future work.

**How Does Dephosphorylation of the MSL10 N-Terminal Domain Trigger Cell Death?**

Though it is not clear under what circumstances MSL10 is dephosphorylated in vivo, our data provide some clues as to the potential mechanisms involved. Inhibition of MSL10’s cell death-promoting activity by phosphorylation was relieved when the soluble N-terminal domain was expressed alone (compare Figures 5 and 6). Perhaps dephosphorylation of the MSL10 N-terminal domain activates its cell death-promoting activity by favoring a structural rearrangement or the removal of a phospho-binding protein (Oecking and Jaspert, 2009; Bozoky et al., 2013; Yamada et al., 2013). According to this explanation, the active conformation would be favored when the soluble N-terminal domain is expressed in the absence of the rest of MSL10. Alternatively, dephosphorylation of the MSL10 N-terminal domain might lead to its cleavage from the rest of the protein, as recently shown for the ER membrane-bound EIN2 in response to ethylene (Ju et al., 2012). The cleaved protein would then be free to activate cell death pathways in the cytoplasm or the nucleus. This would be mimicked by expressing the soluble N-terminal domain on its own, consistent with the results shown in Figure 6. As attractive as this explanation is, we have been unable to obtain direct evidence for it. The lower molecular weight bands seen in MSL10-GFP immunoblots (Figures 1E and 3C) were neither consistently observed nor associated with phosphorylation state. As cleavage could take place anywhere in the soluble N-terminal domain, and could be very inefficient, it remains possible that the cleaved version cannot be distinguished from the wild type or even detected by immunoblot. We attempted to identify cleavage products using a version of MSL10 that was tagged with YFP at the N terminus, but found that it was not properly expressed or trafficked in our transient expression system (Supplemental Figure 7). Thus, MSL10-specific antibodies must be developed in order to more rigorously test the cleavage model.

**What Forms of Cell Death Are Induced by the MSL10 N-Terminal Domain?**

Plant cells can initiate the process of cell death in response to developmental signals, abiotic stress, and pathogenic invasion. Plant cell death can take several forms, in some cases resembling processes that have been identified in mammalian cells such as apoptosis, autophagy, necrosis, and/or senescence (van Doorn and Woltering, 2005; Reape and McCabe, 2008; Coll et al., 2011). The specific pathway activated in *Arabidopsis* or tobacco cells overexpressing MSL10 variants is unclear and might involve one or several of the following cell death mechanisms: activation of nuclear endonucleases, activation of caspase-like or metacaspase proteases, loss of mitochondrial integrity, or targeting of proteins or organelles to the autophagosome (Lam et al., 2001; Coll et al., 2011; Liu and Bassham, 2012). It is notable that ROS production and/or PCD have been documented in response to mechanical stimuli during lateral root emergence (Mergemann and Sauter, 2000; Steffens et al., 2012), osmotic shock response (Okada et al., 2001; Nakayama et al., 2012), immunity (Dodds and...
Rathjen, 2010; Coll et al., 2011; Xin and He, 2013), and abiotic stress (Gill and Tuteja, 2010; Choi et al., 2013), suggesting that ROS-associated programmed cell death is a common response to mechanical stress. While we cannot completely exclude the possibility that the observed phenotypes do not reflect the normal function of MSL10, we can conclude that they are unlikely to be simply the result of ER stress-induced cell death (Tabas and Ron, 2011), as MSL10-GFP accumulated to the same or higher levels as MSL10-GFP and showed similar subcellular localization, but did not appreciably alter cell viability, plant growth, or health.

In conclusion, we have shown that the cytoplasmic N-terminal domain of MSL10 is capable of inducing cell death, that this activity is regulated by phosphorylation, and that MSL10 has therefore two separable functions—one as an ion channel and one as an inducer of cell death. These data provide support for our previous speculations that MSL10 is more than a simple osmotic safety valve. Future studies will help us better understand the diversity of ways in which MS channels have been employed as sensors of membrane tension in plant and bacterial systems.

**METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis thaliana* plants were grown on soil at 21°C under a 24-h light regime (~150 µmol m⁻² s⁻¹). Cell size of adaxial epidermal pavement cells from the fourth youngest leaf of 3-week-old plants was assessed using ImageJ (http://image.nih.gov). For each background, n > 100 cells from five individual plants were counted, two leaves per plant. Transient over-expression of MSL10-GFP variants in tobacco was performed as described (Waadt and Kudla, 2008). For subcellular localization, organelle-specific (PM and ER) markers fused to the fluorophore mCherry (Nelson et al., 2007) were coinfected with bacteria harboring MSL10-GFP and visualized by CLSM 3 d postinfiltration. Tobacco (Nicotiana benthamiana) tissue was subjected to plasmolysis for 5 min in 1 M NaCl to induce Hechtian strands. For the gene expression analysis shown in Supplemental Figure 1, seedlings were grown for 5 d on agar-solidified Murashige and Skoog medium (2 g/L Murashige and Skoog salts [Caisson Labs], pH 5.7, and 0.8% agar [Caisson Labs]) at 21°C under a 16-h light regime.

**Cloning and Transgenic Lines**

All plasmid constructs were made with Gateway technology (Life Technologies). The MSL10 cDNA was cloned previously into pENTR/D-TOPO (Haswell et al., 2008). This clone was used as a template for making all MSL10 variants by the introduction of point mutations via site-directed mutagenesis as described (Jensen and Haswell, 2012). These pENTR constructs were then used in recombination reactions with pK7FWG2 (Karimi et al., 2002) to create variants by the introduction of point mutations via site-directed mutagenesis. The MSL10 cDNA was cloned previously into pENTR/D-TOPO and was collected using a 430- to 470-nm band-pass filter. Images illustrating each of these are shown in Supplemental Figure 1. Cell size of adaxial epidermal pavement cells was quantified using FDA and PI staining procedures as described (Chaves et al., 2002) in *N. benthamiana* leaves by confocal imaging 5 d postinfiltration. Then, leaf samples were immersed in staining solution (500 µg/mL FDA [5 mg/mL stock dissolved in acetonitrile]) and 1.25-µg/mL PI (2.5 mg/mL stock dissolved in water) for 20 min before rinsing with water and imaging of the abaxial epidermis. Images of randomized samples were collected and cell death quantified. Pavement cells were classified as "alive" unless they fulfilled one or more of the following criteria: (1) the presence of PI staining in the nucleus, (2) other PI-staining particles or compartments in the cell center, or (3) the disappearance of a clear vacuole, accompanied by spreading of cytoplasmic GFP/FDA signal. Images illustrating each of these are shown in Supplemental Figure 4. n > 120 cells were counted from each construct from two to three separate infiltration experiments, each consisting of three infiltrated leaves. Statistical differences were analyzed by one-way ANOVA and Tukey’s test, and groups that did not differ significantly were noted (P < 0.05). For DAPI staining, DAPI (5 µg/mL) was dissolved in water with 0.1% Silwet to aid in the penetration of the stain, and leaf samples were floated in the solution for 20 min before imaging. DAPI was excited with a 405-nm laser, and emission was collected using a 430- to 470-nm band-pass filter. For subcellular localization experiments, mCherry was excited with a 543-nm laser and emission was collected using a 560- to 660-nm band-pass filter. Staining with DAB for H₂O₂ production was performed on leaf 4 or 5 from 2-week-old plants as described (Mahalingam et al., 2006). Trypan blue staining for lesion vector pCO2 (Ludewig et al., 2002) between the XbaI and EcoRV sites. The same pENTR constructs containing MSL10 variants were used in recombination experiments with the YFP-containing pPO2 vector.

**RT-PCR**

Whole rosette tissue from 3-week-old *Arabidopsis* was frozen in liquid nitrogen. RNA isolation was performed using Trizol reagent as directed by the manufacturer and 200 ng RNA was used for subsequent cDNA synthesis with oligo(dT) primers and RT-PCR analysis. The oligos used to assess transcriptional activation for each gene are listed in Supplemental Table 2. PCR products were separated on a 2% agarose gel and imaged with ethidium bromide.

**Yeast Two-Hybrid Assays**

Protein-protein interactions were assessed using the mating-based split ubiquitin yeast two-hybrid system as described (Obrdlik et al., 2004). This system was made available through the ABRC, and the procedure was performed essentially as described in the manual provided with the system. Briefly, MSL10 cDNA, either with or without the phospho-point mutations described above, was cloned by homologous recombination into either the THY.AP4 strain, carrying the plasmid pMeryCgate, or the THY.AP5 strain, carrying the plasmid pXYgate33-3HA. The plasmid-containing cells were then mated and diploids were selected on the appropriate dropout media. Interaction was determined by growth after 3 d on minimal media supplemented with 400 µM methionine to decrease background growth.

**Cell Death, Tissue Staining, and Microscopy**

CLSM was performed using a Fluoview FV-1000 (Olympus), and images were captured with FVIO-ASW software (Olympus). Dual staining with FDA and PI was used to quantify cell death (Chaves et al., 2002) in *N. benthamiana* leaf cells transiently expressing MSL10 variants. FDA is a fluorescent indicator of cell viability, as living cells process FDA to produce a fluorescent compound within the cytoplasm (signal excited at 488 nm and emissions collected with a 505- to 525-nm band-pass filter). Preferentially penetrates cells with damaged cell membranes, marking the nucleus and other intracellular compartments in dead cells only (signal excited at 543 nm and emissions collected with a 560- to 660-nm band-pass filter). Before each experiment, MSL10-GFP variant expression throughout the leaf was verified by confocal imaging 5 d postinfiltration. Then, leaf samples were immersed in staining solution (500 µg/mL FDA [5 mg/mL stock dissolved in acetonitrile] and 1.25 µg/mL PI [2.5 mg/mL stock dissolved in water]) for 20 min before rinsing with water and imaging of the abaxial epidermis. Images of randomized samples were collected and cell death quantified. Pavement cells were classified as “alive” unless they fulfilled one or more of the following criteria: (1) the presence of PI staining in the nucleus, (2) other PI-staining particles or compartments in the cell center, or (3) the disappearance of a clear vacuole, accompanied by spreading of cytoplasmic GFP/FDA signal. Images illustrating each of these are shown in Supplemental Figure 4. n > 120 cells were counted from each construct from two to three separate infiltration experiments, each consisting of three infiltrated leaves. Statistical differences were analyzed by one-way ANOVA and Tukey’s test, and groups that did not differ significantly were noted (P < 0.05). For DAPI staining, DAPI (5 µg/mL) was dissolved in water with 0.1% Silwet to aid in the penetration of the stain, and leaf samples were floated in the solution for 20 min before imaging. DAPI was excited with a 405-nm laser, and emission was collected using a 430- to 470-nm band-pass filter. For subcellular localization experiments, mCherry was excited with a 543-nm laser and emission was collected using a 560- to 660-nm band-pass filter. Staining with DAB for H₂O₂ production was performed on leaf 4 or 5 from 2-week-old plants as described (Mahalingam et al., 2006). Trypan blue staining for lesion vector pCO2 (Ludewig et al., 2002) between the XbaI and EcoRV sites. The same pENTR constructs containing MSL10 variants were used in recombination experiments with the YFP-containing pPO2 vector.
formation was performed on leaf number 4 or 5 on 3-week-old plants as described (Vogel and Somerville, 2000).

**Oocyte Preparation**

Oocytes from *X. laevis* (Dumont stage V or VI) females were isolated as previously described (Yang and Sachs, 1990; Stühmer and Parekh, 1995) and incubated in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, and 5 mM HEPES, pH 7.4) with 50 mg/mL gentamicin at 18°C overnight. The day after isolation, cells were injected with 50 nL of 1 μg/μL cRNA and were patched 7 to 14 d after injection. Prior to patching, vitelline membranes were removed from the oocytes with a pair of dull forceps.

**Isolation of Oocyte Membrane Fractions**

Isolation of *X. laevis* oocyte membranes was performed essentially as described (Dixit et al., 2001). Briefly, 20 devitelilized oocytes expressing wild-type MSL10-GFP variants were placed into 1 mL of the following solution: 7.5 mM Na$_2$HPO$_4$, 1 mM EDTA, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 μg/mL pepstatin and ground with a pestle. The tube was then spun at ~750 g for 5 min at 4°C. The supernatant fraction was transferred to a new tube and spun for 30 min at ~16,000 g at 4°C. The pellet was resuspended in 40 μL of loading buffer with β-mercaptoethanol, flash-frozen in liquid nitrogen, and stored at ~80°C. Aliquots equivalent to one oocyte were used for each lane in immunoblot analysis.

**Electrophysiology**

Devitelilized oocytes were patched in symmetric modified ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 10 mM MgCl$_2$, and 5 mM HEPES, pH 7.4) using pipette bubble number 4.5-5 in the inside-out (excised) patch configuration. Pressure ramps were generated by a High Speed Pressure System (HSPS-1; ALA Sciences). The micromanipulator system Scientifica PatchStar 700 was used for membrane patching (Scientifica). Data were acquired at 20 kHz, filtered at 5 kHz, and digitized with the Axopatch 200B patch-clamp amplifier (Molecular Devices) and further analyzed with the pClamp10 software suite (Molecular Devices). Pipettes were fabricated of Kimax 51 patch glass (Kimble Products) using a Sutter P-97 puller (Sutter Instruments). Measurements were made on at least three oocytes for each wild-type protein and each mutant selected from at least two different cultures. Recordings were made at ~30 to ~40 mV membrane potentials and ~40 to ~200 mmHg transmembrane pressures, while mechanosensitive activity typically began to appear at ~60 to ~70 mm Hg pressure.

**Immunoblot Analysis**

Total plant protein ground in 2× sample buffer was used for immunoblot analysis. The proteins were denatured for 5 min at 97°C, separated by SDS-PAGE (100 V for 2.5 h), and transferred to a polyvinylidene fluoride membrane. After overnight blocking in TBST (10 mM Tris-Cl, 150 mM NaCl, and 0.05% [v/v] Tween 20, pH 7.5) with 5% (w/v) nonfat milk powder, the membrane was incubated for 1 h with primary anti-GFP antibody (Clontech) at room temperature. Then the membrane was treated with anti-mouse secondary antibodies for 1 h at room temperature. Detection was performed using a Thermo Scientific Femto Detection Kit (Thermo Scientific) and BioMax XAR film (Kodak). Blots were subsequently stripped and reprobed as above with anti-α-tubulin antibody (Sigma-Aldrich) to assess loading.

**Molecular Dynamics Simulations**

The initial structure of the MSL10 N terminus (residues 1 to 167) was derived from MODELER (Andrej Sali Lab, USCF) online server predictions. It was simulated in a water environment (including ions equivalent to 100 mM) at 27°C using the GROMACS package (Royal Institute of Technology and Uppsala University, Sweden) for 140 ns until it stabilized. Serines at positions 29, 46, 48, 57, 128, 131, and T136 in the MSL10 N-terminal domain were replaced with alanines (MSL10_A1-167) or aspartic/glutamic acid (MSL10_D1-167, as only residue 131 was mutated to Glu) using the “Mutator” VMD package tool (University of Illinois at Urbana-Champaign). Each of the structures (MSL10, MSL10_A1-167, and MSL10_D1-167) was simulated under the same conditions for an additional 100 ns. The results of these simulations are presented as images generated by the DSSP plug-in for GROMACS (Kabsch and Sander, 1983)

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT5G12080, AT5G45890, AT4G11650, AT3G01420, AT3G49120, and AT1G73260.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Additional Phenotypes Associated with MSL10-GFP Overexpression.

**Supplemental Figure 2.** Multiple Sequence Alignment of the N Termini of MSL10 Orthologs in Land Plants.

**Supplemental Figure 3.** Multiple Sequence Alignment of Class II MSL Proteins from *Arabidopsis thaliana* and *MscS* from *Escherichia coli*.

**Supplemental Figure 4.** Documentation of the Cell Death Assay used in Figures 3, 5, and 6.

**Supplemental Figure 5.** Electrophysiological Analysis of MSL10 Variants in Xenopus Oocytes.

**Supplemental Figure 6.** Molecular Dynamics Simulations of the Soluble N-Terminal Domain of MSL10.

**Supplemental Figure 7.** Subcellular Localization of N-Terminally Tagged Full-Length YFP-MSL10 Variants.

**Supplemental Table 1.** Experimentally Determined in Vivo Phosphorylated Peptides Attributed to the MSL10 N Terminus.

**Supplemental Table 2.** Primers Used for RT-PCR.

**Supplemental References**

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**AUTHOR CONTRIBUTIONS**

K.M.V., G.M., and E.S.H. wrote the article, performed and designed experiments, and conducted data analysis. E.M.F. performed and designed experiments, and conducted data analysis. E.J. and S.C.K. performed experiments and conducted data analysis. Received May 23, 2014; revised June 19, 2014; accepted June 27, 2014; published July 22, 2014.
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Arabidopsis MSL10 Has a Regulated Cell Death Signaling Activity That Is Separable from Its Mechanosensitive Ion Channel Activity
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