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Mechanosensitive channel MSL8 regulates osmotic forces during pollen hydration and germination

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Abstract

Pollen grains undergo dramatic changes in cellular water potential as they deliver the male germ line to female gametes, and it has been proposed that mechanosensitive ion channels may sense the resulting mechanical stress. Here, we identify and characterize MscS-like 8 (MSL8), a pollen-specific, membrane tension-gated ion channel required for pollen to survive the hypoosmotic shock of rehydration and for full male fertility. MSL8 negatively regulates pollen germination but is required for cellular integrity during germination and tube growth. MSL8 thus senses and responds to changes in membrane tension associated with pollen hydration and germination. These data further suggest that homologs of bacterial MscS have been repurposed in eukaryotes to function as mechanosensors in multiple developmental and environmental contexts.

Mechanosensitive (stretch-activated) ion channels provide an evolutionarily conserved mechanism for the perception of mechanical force at the membrane (1). The mechanosensitive channel of small conductance (MscS) from *Escherichia coli* belongs to a large and structurally diverse family of proteins encoded in bacterial, archaeal, plant, and fungal genomes (2, 3). Bacterial MscS homologs prevent cellular lysis upon hypoosmotic shock by releasing osmolytes from the cell in direct response to increased lateral membrane tension (4). MscS-like (MSL) proteins in plants exhibit homology to the pore-lining domain of *E. coli* MscS; outside of this region, they show diverse domains and topologies (3) (fig. S1, A and B). *Arabidopsis thaliana* mutants lacking functional *MSL* genes respond normally to externally applied osmotic or mechanical stresses (5).

We therefore hypothesized that MscS homologs in plants may sense and respond to rapid changes in water status (and therefore membrane tension) that are intrinsic to the plant life cycle rather than environmentally imposed. Several such events occur during the development of pollen, the multicellular haploid life stage of plants that harbors the male

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S8

Table S1

References (26–32)

gametes (6). In most angiosperms, including *A. thaliana*, the last stage of pollen maturation is partial dehydration (<30% water content) (7). Once the desiccated pollen grain contacts the stigma cells of a compatible female flower, stigma exudate enters the grain and reactivates its metabolism (8). The pollen tube germinates, breaking through the grain cell wall and proceeding via polarized tip growth toward female gametes inside the ovaries (9). The mechanical stress exerted on pollen membranes and cell walls (10, 11) and the spatially and temporally dynamic ion fluxes known to be essential for pollen grain germination and tube growth (12) suggest a role for stretch-activated ion channels (13). Mechanosensitive cation channel activities have been detected in pollen grain and tube membranes (14), but their molecular identity and physiological functions remain unknown.

A. thaliana *MSL8* (At2g17010) transcripts were detected in mRNA isolated from floral but not leaf or root tissue (fig. S1C). In transgenic plants expressing genomic *MSL8* fused to green fluorescent protein (*gMSL8-GFP*) under the control of native sequences, fluorescence was observed inside half of the pollen grains within the anthers of the hemizygous first transformed T1 generation (Fig. 1A). *gMSL8-GFP* signal was observed in tricellular and mature pollen (Fig. 1, B to F) (6) but not in any other tissue. *MSL8* transcripts were identified in an RNA-sequencing data set from mature, dry pollen (15) (fig. S1D). Phylogenetic analysis suggests that male-specific expression of *MSL* genes evolved in both monocot and dicot lineages (fig. S2).

MSL8-GFP expressed from endogenous sequences localized both to the plasma membrane and to endomembrane compartments in pollen grains, and upon germination was mobilized to the tube periphery (Fig. 1, G and H), as did *MSL8-YFP* (yellow fluorescent protein) expressed from the strong pollen-specific promoter *LAT52* (Fig. 1I) (16). *MSL8-YFP* colocalized with the pollen plasma membrane protein CPK34 (17) but not with an endoplasmic reticulum marker (maximum Pearson's correlation coefficients of 0.66 and 0.09, respectively) (Fig. 1, J to L, and fig. S3), and there was no substantial overlap with Golgi or vacuole markers (fig. S4). A similar internal localization pattern has been observed with other pollen plasma membrane proteins (18, 19).

MSL8 produced mechanosensitive ion channel activity when expressed in *Xenopus laevis* oocytes (Fig. 2A and fig. S5, A and B). In this system, *MSL8* (or *MSL8-YFP*, which was indistinguishable) (fig. S5C) had a unitary conductance of 57 pS under negative membrane potentials and 39 pS under positive membrane potentials (Fig. 2B); the conductance of *MscS* is ~300 pS under similar conditions (20). *MSL8* exhibited a 6.3-fold preference for chloride over sodium (Fig. 2C) and is therefore more anion-selective than *MscS*, which has a permeability ratio ($P_{Cl}:P_K$) of 1.2 to 3.0 (21). Finally, the gating threshold pressure for *MSL8* was higher than that of *MscS* (-48.2 ± 14.8 mm Hg and -19.1 ± 5.1 mm Hg, respectively; $n = 9$ patches) (fig. S5, D and E). *MSL8* activity was unaffected by treatment with $MgCl_2$, ruthenium red, or tetramethylammonium-Cl (figs. S5C and S6, A to C). A mechanosensitive channel activity with conductance similar to *MSL8* under the same conditions was occasionally detected in wild-type *Col-0* pollen protoplasts (5 of 58 patches) and may correspond to the endogenous *MSL8* channel (Fig. 2D and fig. S6, D to F). Final confirmation will require demonstrating the loss of channel activity in *msl8* mutant pollen grains.

Two transfer DNA (T-DNA) insertion alleles were identified that resulted in the reduction and loss of detectable *MSL8* transcripts in the flower, *msl8-1* and *msl8-4*, respectively (fig. S7, A and B). The null *msl8-4* allele was transmitted through the male germ line with reduced efficiency, while it was transmitted normally through the female germ line (Fig. 3A). Even modest transmission defects will result in rapid purification from a natural population, as pollen-specific genes are subject to strong selection against deleterious mutations (22). We detected no obvious morphological defects in the coat or cell wall of desiccated *msl8-4* pollen grains (Fig. 3B).

To test the hypothesis that *MSL8* is required for pollen to survive the osmotic downshift experienced during rehydration, we quantified the viability of mature pollen after rehydration in distilled water. Whereas wild-type pollen exhibited 83 to 95% viability over the 2-hour time course, *msl8-4* pollen viability dropped from 38% to 21%, and *msl8-1* pollen viability dropped from wild-type levels to 46% (Fig. 3C–D). This phenotype was rescued by the *gMSL8-GFP* transgene in both mutant backgrounds (fig. S7, C and D).

While the osmotic shock of in vitro hydration in distilled water is more extreme than pollen grains are likely to experience in vivo, *msl8-4* pollen also shows a defect in viability when hydrated in low concentrations of polyethylene glycol (PEG) 3350 (Fig. 3E). These results are consistent with previous work showing that even slow rates of osmotic downshock are lethal to an *E. coli* strain lacking mechanosensitive ion channels (23). Increasing the osmolarity of the hydration medium led to corresponding increases in pollen viability. The in vivo hydration rate of *msl8-4* pollen did not differ from the wild type (fig. S7E). Finally, *msl8-4* pollen grains dissected from anthers before desiccation showed wild-type viability upon hydration (Fig. 3F). Thus, *msl8* pollen developed normally and was fully viable before dehydration, and its loss of viability when hydrated can be attributed to the hypoosmotic challenge of water entering the desiccated grain.

MSL8 also plays a role in pollen germination. During in vitro germination assays, *msl8-4* pollen grains and germinated tubes burst 26% of the time, whereas only 3% of the wild type burst (Fig. 4, A to C). Pollen bursting is associated with cell-wall defects and lesions in ion channel genes and is thus thought to result from a failure to balance osmotic pressure with the strength of the cell wall (19). *msl8-4* and *msl8-1* pollen germinated at a higher rate than the corresponding wild type (both burst and intact tubes: 43% in *msl8-4* and 57% in *msl8-1*; compare to 20% and 23% in Ler and Col-0, respectively) (Fig. 4, A to C). Conversely, overexpression of *LAT52pMSL8-YFP* inhibited germination, and three independent homozygous lines exhibited only 4 to 39% of wild-type germination rates (Fig. 4D). The germination rate was inversely correlated with the level of *MSL8-YFP* transcript in these lines (Fig. 4E), confirming that *MSL8* negatively regulates in vitro germination. *MSL8-YFP* overexpression lines were impaired in transmission of the transgene to the next generation (Fig. 4F and fig. S8A), but only through the male parent (Fig. S8B–C). Because these lines exhibited wild-type hydration survival (fig. S8D), this defect can be attributed to reduced rates of pollen germination.

Changes in osmotic potential are part of normal pollen function, in addition to being environmental stresses that must be tolerated. Here, we show that *MSL8* is required for a

tuned response to developmentally normal osmotic challenges. During pollen rehydration, MSL8 maintains cellular integrity upon osmotic downshift, playing a role analogous to that of *E. coli* MscS. During pollen germination, however, MSL8 maintains the optimal osmotic potential required to drive germination yet prevent lysis of the nascent pollen tube. MSL8 may accomplish these functions by releasing osmolytes directly in response to membrane tension and/or function indirectly in pathways that regulate pollen desiccation, membrane trafficking, or cell-wall dynamics. This study illustrates how MSL8, a eukaryotic homolog of the bacterial osmotic safety valve MscS, has been re-purposed to help pollen cope with predictable osmotic changes that are characteristic of pollen development. It also contributes to a growing body of evidence that mechanical signaling plays a critical role in plant and animal development (24, 25).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 2. MSL8 forms a small-conductance mechanosensitive ion channel with a preference for anions that is similar to a channel present in pollen membranes

(A) Representative trace from an excised inside-out patch of plasma membrane from a *Xenopus laevis* oocyte expressing *MSL8-YFP* capped RNA (cRNA) (left) or water-injected (right) at -60 mV membrane potential. Channels were gated by negative tension (suction) applied to the patch pipette. The first three channel openings are labeled O₁ to O₃. (B) The current-voltage relationship of untagged tension-gated MSL8 in symmetric ND96 buffer. *N* = 8 oocytes. (C) The current-voltage relationship of tension-gated MSL8 under symmetric (filled circles, 100 mM NaCl) and asymmetric (open circles, 100 mM NaCl pipette/300 mM NaCl bath) conditions. *N* = 3 oocytes. (D) Representative trace from an excised inside-out patch of membrane from a Col-0 pollen protoplast at a transmembrane potential of -60 mV. Inset, four channel openings with characteristics similar to MSL8 are labeled O₁ to O₄.

Fig. 3. MSL8 is required for mature pollen grains to survive hypoosmotic shock during rehydration
(A) Transmission ratio analysis of the *msl8-4* allele. The progeny of reciprocal crosses between *msl8-4* heterozygotes and wild-type plants were genotyped. *P* values were determined by a chi-square test against the expected ratio of 50:50. (B) Scanning electron micrograph of desiccated pollen from the indicated genotypes. Scale bar is 20 μ m. (C) Viability of wild-type and mutant pollen after hydration. Pollen was incubated for 30 min in distilled water containing fluorescein diacetate and propidium iodide, dyes that stain viable and unviable pollen, respectively. Asterisks mark compromised pollen. Scale bar, 100 μ m. (D) Hydration viability time course. The average of three experiments with *N* = 55170 pollen grains is shown. Error bars, mean $\pm$ SD. Asterisks indicate significant (*P* < 0.05) differences from the wild type by Student's *t* test. (E) Effect of a PEG series on viability (bars, 3 to 5 trials per genotype; *N* = 48 to 440 pollen grains per trial; error bars, mean $\pm$ SE) and pollen diameter (lines, *N* = 15 pollen grains per genotype; error bars, mean $\pm$ SD) after hydration. (F) Viability after hydration of nondehiscent tricellular pollen grains dissected from the anther of the indicated genotypes. *N* > 50 pollen grains per genotype. Error bars, mean $\pm$ SD.

Fig. 4. *MSL8* negatively regulates germination and is required for cellular integrity during germination

Wild-type (A) and *msl8-4* (B) pollen germinated for 4 hours in liquid germination media and stained with aniline blue for callose, a marker of germination. Examples of ungerminated (single asterisk), ungerminated and burst (double asterisk), germinated (white arrow), or germinated and burst (black arrow) pollen are indicated. Scale bar, 50 μ m. (C) Germination rate and bursting frequency in the indicated genotypes. $N = 396$ pollen grains per genotype. Asterisks indicate significant ($P < 0.05$) differences by Student's t test (D) Percentage germination overnight on solid media of pollen from wild-type and three independent homozygous *LAT52pMSL8-YFP* transgenic lines. Error bars, mean $\pm$ SE. (E) Quantitative reverse-transcription polymerase chain reaction of *MSL8-YFP* and *MSL8* transcripts relative to *ACTIN* in flowers from Ler, *msl8-4*, and the *LAT52pMSL8-YFP* lines in (D). Two technical replicates of three biological replicates are presented. Error bars, mean $\pm$ SE. (F) Survival of selection for the Bialaphos resistance gene in offspring of the *LAT52pMSL8-YFP* lines in (D). P values were determined by a chi-square test against 75% expected survival.