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10

11   **Abstract:** Pollen grains undergo dramatic changes in cellular water potential as they deliver the male

12   germ line to female gametes, and it has been proposed that mechanosensitive ion channels may sense the

13   resulting mechanical stress. Here we identify and characterize MscS-Like (MSL)8, a pollen-specific,

14   membrane tension-gated ion channel required for pollen to survive the hypoosmotic shock of rehydration

15   and for full male fertility. MSL8 negatively regulates pollen germination, but is required for cellular

16   integrity during germination and tube growth. MSL8 thus senses and responds to changes in membrane

17   tension associated with pollen hydration and germination. These data further suggest that homologs of

18   bacterial MscS have been repurposed in eukaryotes to function as mechanosensors in multiple

19   developmental and environmental contexts.

20

21

22   **Summary:** A plant homolog of the bacterial mechanosensitive ion channel MscS is required to respond

23   to multiple osmotic challenges during pollen hydration and germination.

24

25   Mechanosensitive (stretch-activated) ion channels provide an evolutionarily conserved mechanism for the

26   perception of mechanical force at the membrane (*1*). The <u>M</u>echano<u>s</u>ensitive <u>c</u>hannel of <u>S</u>mall conductance

27   (MscS) from *Escherichia coli* belongs to a large and structurally diverse family of proteins encoded in

28   bacterial, archaeal, plant, and fungal genomes (*2, 3*). Bacterial MscS homologs prevent cellular lysis upon

29   hypoosmotic shock by releasing osmolytes from the cell in direct response to increased lateral membrane

30   tension (*4*). <u>MscS</u>-<u>L</u>ike *(MSL)* proteins in plants exhibit homology to the pore-lining domain of *E. coli*

31   MscS; outside of this region they show diverse domains and topologies ((*3*), Fig. S1A, B). *Arabidopsis*

32   *thaliana* mutants lacking functional *MSL* genes respond normally to externally applied osmotic or

33   mechanical stresses (*5*).

34

35   We therefore hypothesized that MscS homologs in plants may sense and respond to rapid changes in

36   water status (and therefore membrane tension) that are intrinsic to the plant life cycle rather than

37   environmentally imposed. Several such events occur during the development of pollen, the multicellular

38   haploid life stage of plants that harbors the male gametes (*6*). In most angiosperms, including *A. thaliana,*

39   the last stage of pollen maturation is partial dehydration (< 30% water content) (*7*). Once the desiccated

40   pollen grain contacts the stigma cells of a compatible female flower, stigma exudate enters the grain and

41   reactivates its metabolism (*8*). The pollen tube germinates, breaking through the grain cell wall and

42   proceeding via polarized tip growth toward female gametes inside the ovaries (*9*). The mechanical stress

43   exerted on pollen membranes and cell walls (*10, 11*) and the spatially and temporally dynamic ion fluxes

44   known to be essential for pollen grain germination and tube growth (*12*) suggest a role for stretch-

45   activated ion channels (*13*). Mechanosensitive cation channel activities have been detected in pollen grain

46   and tube membranes (*14*), but their molecular identity and physiological functions remain unknown.

47

48   *A. thaliana MSL8* (At2g17010) transcripts were detected in mRNA isolated from floral but not leaf or

49   root tissue (Fig. S1C). In transgenic plants expressing genomic *MSL8* fused to *Green Fluorescent Protein*

50   (*gMSL8-GFP*) under the control of native sequences, fluorescence was observed inside half of the pollen

51   grains within the anthers of the hemizygous first transformed T1 generation (Fig. 1A). *gMSL8-GFP*

52   signal was observed in tricellular and mature pollen (Fig. 1B-F, (*6*)), but not in any other tissue. *MSL8*

53   transcripts were identified in an RNA-Seq dataset from mature, dry pollen (*15*) (Fig. S1D). Phylogenetic

54   analysis suggests that male-specific expression of *MSL* genes evolved in both monocot and dicot lineages

55   (Fig. S2).

56

57   MSL8-GFP expressed from endogenous sequences localized both to the plasma membrane and to

58   endomembrane compartments in pollen grains, and upon germination was mobilized to the tube periphery

59   (Fig. 1G-H), as did MSL8-YFP expressed from the strong pollen-specific promoter *LAT52* (Fig. 1I) (*16*).

60   MSL8-YFP colocalized with the pollen plasma membrane protein CPK34 (*17*) but not with an

61 endoplasmic reticulum marker (maximum Pearson's correlation coefficients of 0.66 and 0.09,

62 respectively; Fig. 1J-L, Fig. S3), and there was no substantial overlap with Golgi or vacuole markers (Fig.

63 S4). A similar internal localization pattern has been observed with other pollen plasma membrane

64 proteins (*18, 19*).

65

66 MSL8 produced mechanosensitive ion channel activity when expressed in *Xenopus laevis* oocytes (Figs.

67 2A, S5A-B). In this system, MSL8 (or MSL8-YFP, which was indistinguishable, Fig. S5C) had a unitary

68 conductance of 57 pS under negative membrane potentials and 39 pS under positive membrane potentials

69 (Fig. 2B); the conductance of MscS is ~300 pS under similar conditions (*20*). MSL8 exhibited a 6.3-fold

70 preference for chloride over sodium (Fig. 2C), and is therefore more anion-selective than MscS, which

71 has a $P_{Cl} : P_K$ ratio of 1.2 - 3.0 (*21*). Finally, the threshold tension for MSL8 higher than MscS (-48.2 ±

72 14.8 mm Hg and -19.1 ± 5.1 mm Hg, respectively (n = 9; Fig. S5D-E)). MSL8 activity was unaffected by

73 treatment with $MgCl_2$, ruthenium red or tetramethylammonium-Cl (Figs. S5C, S6A-C). A

74 mechanosensitive channel activity with conductance similar to MSL8 under the same conditions was

75 occasionally detected in wild type Col-*0* pollen protoplasts (5/58 patches), and may correspond to the

76 endogenous MSL8 channel (Fig. 2D, S6D-F). Final confirmation will require demonstrating the loss of

77 channel activity in *msl8* mutant pollen grains.

78

79 Two T-DNA insertion alleles were identified that resulted in the reduction and loss of detectable *MSL8*

80 transcripts in the flower, *msl8-1* and *msl8-4*, respectively (Fig. S7A-B). The null *msl8-4* allele was

81 transmitted through the male germline with reduced efficiency, while it was transmitted normally through

82 the female germline (Fig. 3A). Even modest transmission defects will result in rapid purification from a

83 natural population, as pollen-specific genes are subject to strong selection against deleterious mutations

84 (*22*). We detected no obvious morphological defects in the coat or cell wall of desiccated *msl8-4* pollen

85 grains (Fig. 3B).

86

87 To test the hypothesis that *MSL8* is required for pollen to survive the osmotic downshift experienced

88 during rehydration, we quantified the viability of mature pollen after rehydration in distilled water. While

89 wild type pollen exhibited 83-95% viability over the 2-hour time course, *msl8-4* pollen viability dropped

90 from 38% to 21% and *msl8-1* pollen viability dropped from wild type levels to 46% (Fig. 3C-D). This

91 phenotype was rescued by the *gMSL8-GFP* transgene in both mutant backgrounds (Fig. S7C-D).

92

93 While the osmotic shock of *in vitro* hydration in distilled water is more extreme than pollen grains are

94 likely to experience in vivo, *msl8-4* pollen also shows a defect in viability when hydrated in low

95 concentrations of polyethylene glycol (PEG) 3350 (Fig. 3E). These results are consistent with previous

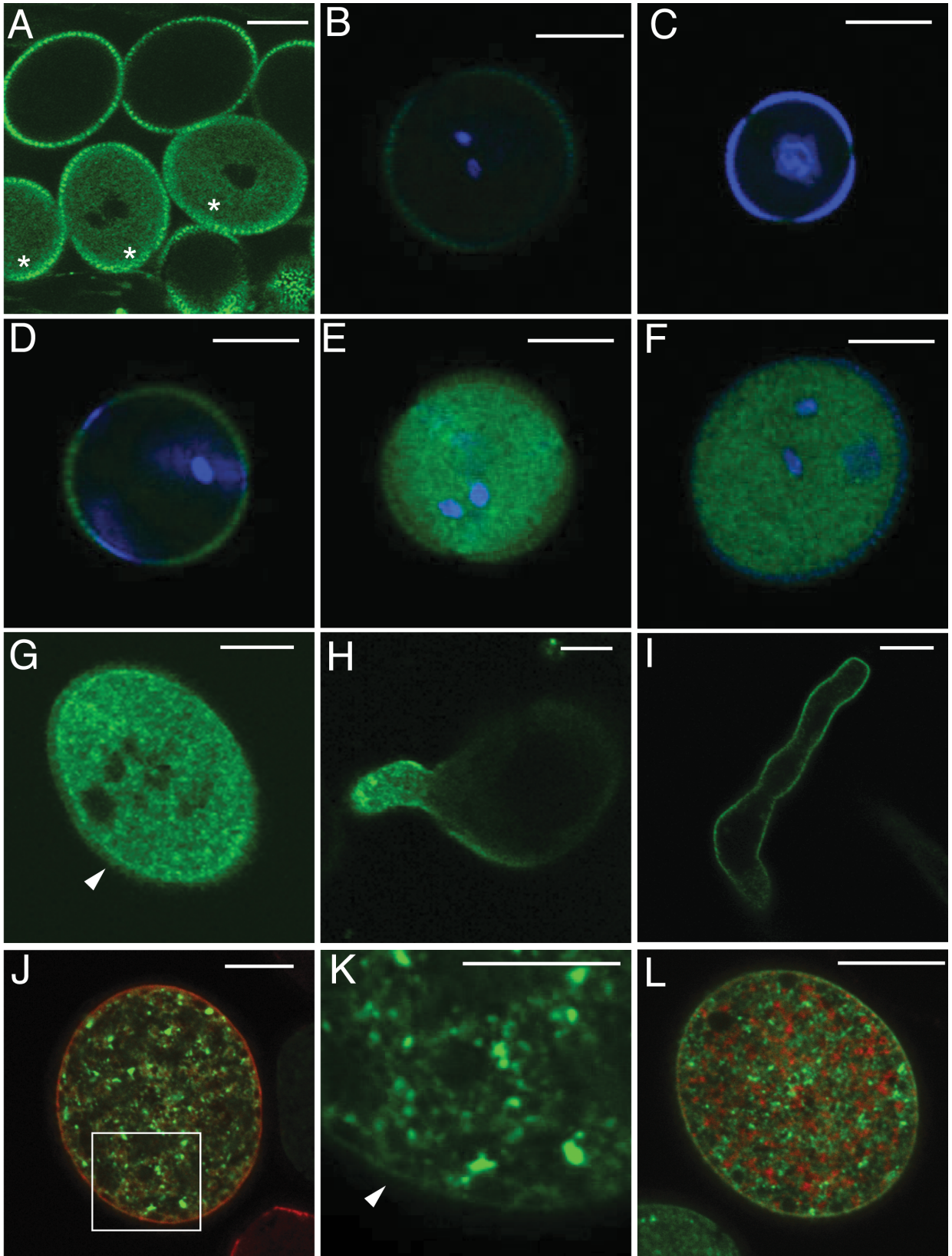96 work showing that even slow rates of osmotic downshock are lethal to an *E. coli* strain lacking

97   mechanosensitive ion channels (*23*). Increasing the osmolarity of the hydration medium led to

98   corresponding increases in pollen viability. The *in vivo* hydration rate of *msl8-4* pollen did not differ from

99   the wild type (Fig. S7E). Finally, *msl8-4* pollen grains dissected from anthers prior to desiccation showed

100   wild-type viability upon hydration (Fig. 3F). Thus, *msl8* pollen developed normally and was fully viable

101   before dehydration, and its loss of viability when hydrated can be attributed to the hypoosmotic challenge

102   of water entering the desiccated grain.

103

104   MSL8 also plays a role in pollen germination. During *in vitro* germination assays, *msl8-4* pollen grains

105   and germinated tubes burst 26% of the time while only 3% of the wild type burst (Fig. 4A-C). Pollen

106   bursting is associated with cell wall defects and lesions in ion channel genes, and is thus thought to result

107   from a failure to balance osmotic pressure with the strength of the cell wall (*19*). *msl8-4 and msl8-1*

108   pollen germinated at a higher rate than the corresponding wild type (both burst and intact tubes: 43% in

109   *msl8-4* and 57% in *msl8-1*, compare to 20% and 23% in L*er* and Col-*0*, respectively, Fig. 4A-C).

110   Conversely, over-expression of *LAT52pMSL8-YFP* inhibited germination, and three independent

111   homozygous lines exhibited only 4-39% of wild type germination rates (Fig. 4D). The germination rate

112   was inversely correlated with the level of *MSL8-YFP* transcript in these lines (Fig. 4E), confirming that

113   MSL8 negatively regulates *in vitro* germination. *MSL8-YFP* over-expression lines were impaired in

114   transmission of the transgene to the next generation (Fig. 4F, Fig. S8A), but only through the male parent

115   (Fig. S8B-C). As these lines exhibited wild type hydration survival (Fig. S8D), this defect can be

116   attributed to reduced rates of pollen germination.

117

118   Changes in osmotic potential are part of normal pollen function, in addition to being environmental

119   stresses that must be tolerated. Here we show that MSL8 is required for a tuned response to

120   developmentally normal osmotic challenges. During pollen rehydration, MSL8 maintains cellular

121   integrity upon osmotic downshift, playing a role analogous to that of *E. coli* MscS. During pollen

122   germination, however, MSL8 maintains the optimal osmotic potential required to drive germination yet

123   prevent lysis of the nascent pollen tube. MSL8 may accomplish these functions by releasing osmolytes

124   directly in response to membrane tension and/or function indirectly in pathways that regulate pollen

125   desiccation, membrane trafficking or cell wall dynamics. This study illustrates how MSL8, a eukaryotic

126   homolog of the bacterial osmotic safety valve MscS, has been repurposed to help pollen cope with

127   predictable osmotic changes that are characteristic of pollen development. It also contributes to a growing

128   body of evidence that mechanical signaling plays a critical role in plant and animal development (*24, 25*).

129

130

138   **Fig. 1. MSL8 is expressed in mature pollen grains and localizes to the plasma membrane and**

139   **endomembranes of pollen grains and germinating tubes.** (**A**) Confocal image of GFP signal in a

140   dissected anther from stage 12 flowers of a *gMSL8-GFP* T1 plant. Asterisks mark transgenic pollen. The

141   pollen cell wall is auto-fluorescent, but internal fluorescence is ascribed to MSL8-GFP. (**B-F**) Confocal

142   images of GFP (green) and DAPI signal (blue) during pollen development. (**B**) Tricellular pollen from a

143   non-transformed line. Pollen was isolated from a *gMSL8-GFP*-expressing line at the (**C**) microspore, (**D**)

144   bicellular, (**E**) tricellular, and (**F**) mature stages of development. (**G-H**) Confocal images of ungerminated

145   (**G**) and *in vivo* germinated (**H**) pollen grains in a *gMSL8-GFP*-expressing line. (**I**) A germinated pollen

146   tube from a line expressing *LAT52pMSL8-YFP*. (**J**) MSL8-YFP (green) overlaid with CPK34-mCherry

147   (red). (**K**) Magnification of box indicated in (**J**), green channel only. Arrow indicates plasma membrane

148   (**L**) MSL8-YFP overlaid with endoplasmic reticulum-mCherry (red). Scale bars are 10 (**B-J, L**) or 5 μm

149   (**K**).
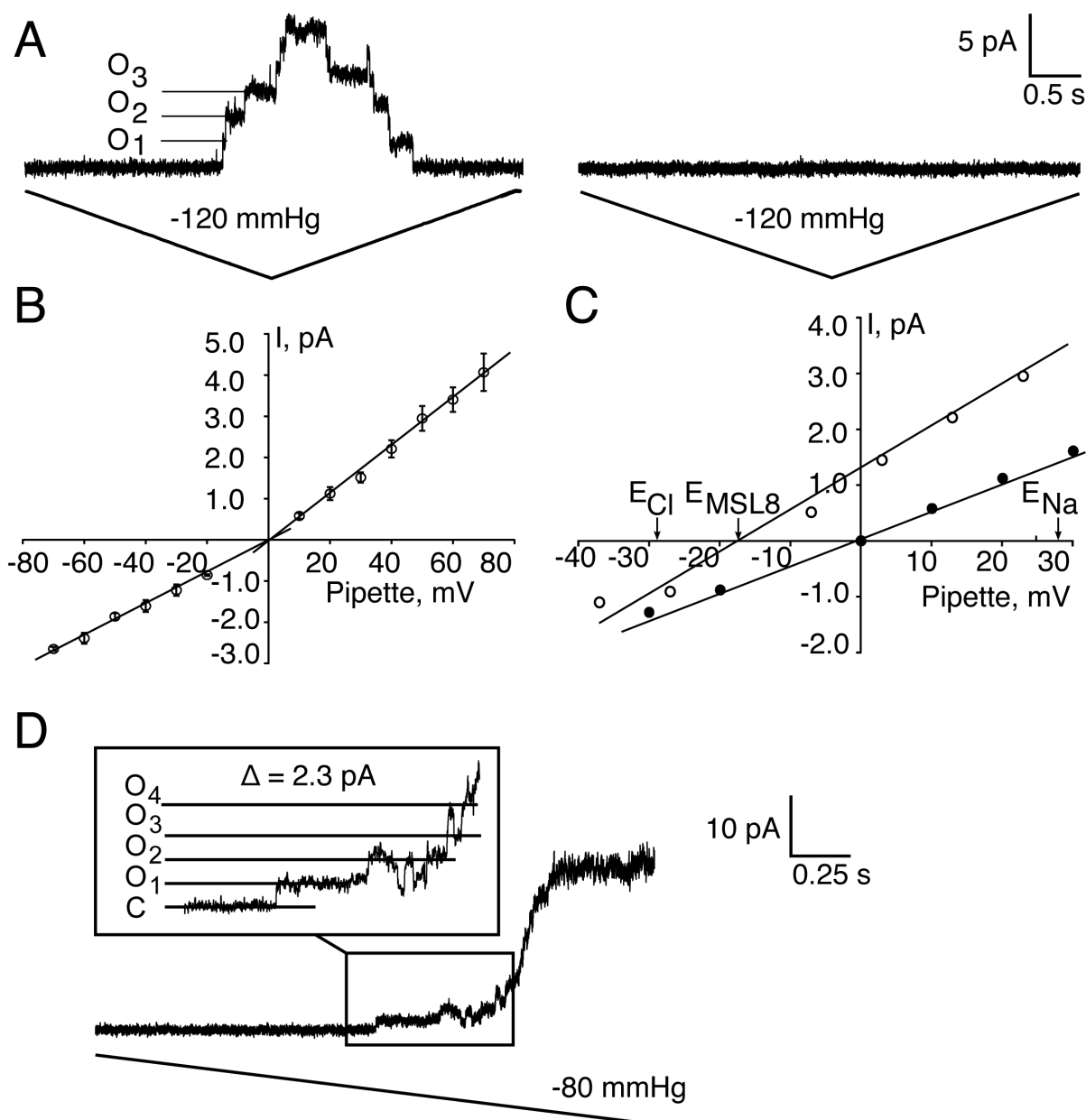
Fig. 2. MSL8 forms a small-conductance mechanosensitive ion channel with a preference for anions that is similar to a channel present in pollen membranes. Representative trace from an excised inside-out patch of plasma membrane from a *Xenopus laevis* oocyte expressing *MSL8-YFP* cRNA (**left**) or water-injected (**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 O_1 to O_3. (**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

160 transmembrane potential of -60 mV. Inset, four channel openings with characteristics similar to MSL8 are

161 labeled O_1-O_4.

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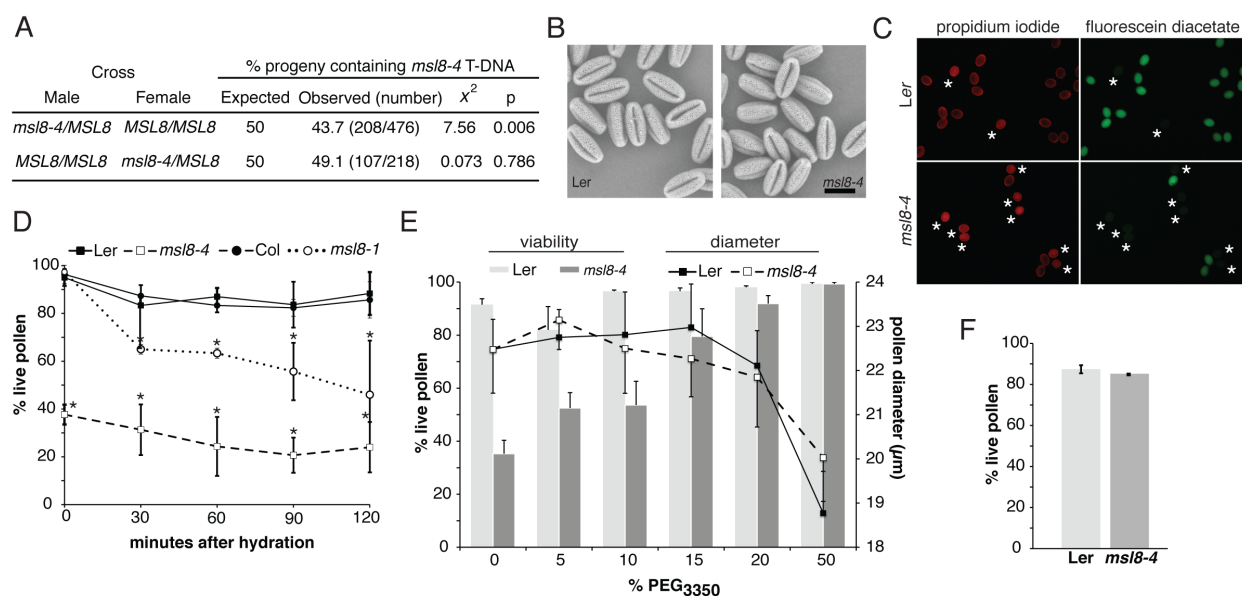
164

165 **Fig. 3. MSL8 is required for mature pollen grains to survive hypoosmotic shock during rehydration.**

166 (**A**) Transmission ratio analysis of the *msl8-4* allele. The progeny of reciprocal crosses between *msl8-4*

167 heterozygotes and wild type plants were genotyped. P-values were determined by a chi-squared test

168 against the expected ratio of 50:50. (**B**) Scanning electron micrograph of desiccated pollen from the

169 indicated genotypes. Scale bar is 20 μm. (**C**) Viability of wild type and mutant pollen after hydration.

170 Pollen was incubated for 30 minutes in distilled water containing fluorescein diacetate and propidium

171 iodide, dyes that stain viable and unviable pollen respectively. Asterisks mark compromised pollen. (**D**)

172 Hydration viability time course. The average of three experiments with N = 55-170 is shown. Error bars

173 indicate standard deviation. Asterisks indicate significant (p < 0.05) differences from the wild type by

174 Student's *t*-test. (**E**) Effect of a PEG series on viability (bars, 3-5 trials per genotype, N = 48-440 per trial,

175 error bars are standard error) and pollen diameter (lines, N = 15, error bars are standard deviation) after

176 hydration. (**F**) Viability after hydration of nondehiscent tricellular pollen grains dissected from the anther

177 of the indicated genotypes. N > 50. Error bars indicate standard deviation.
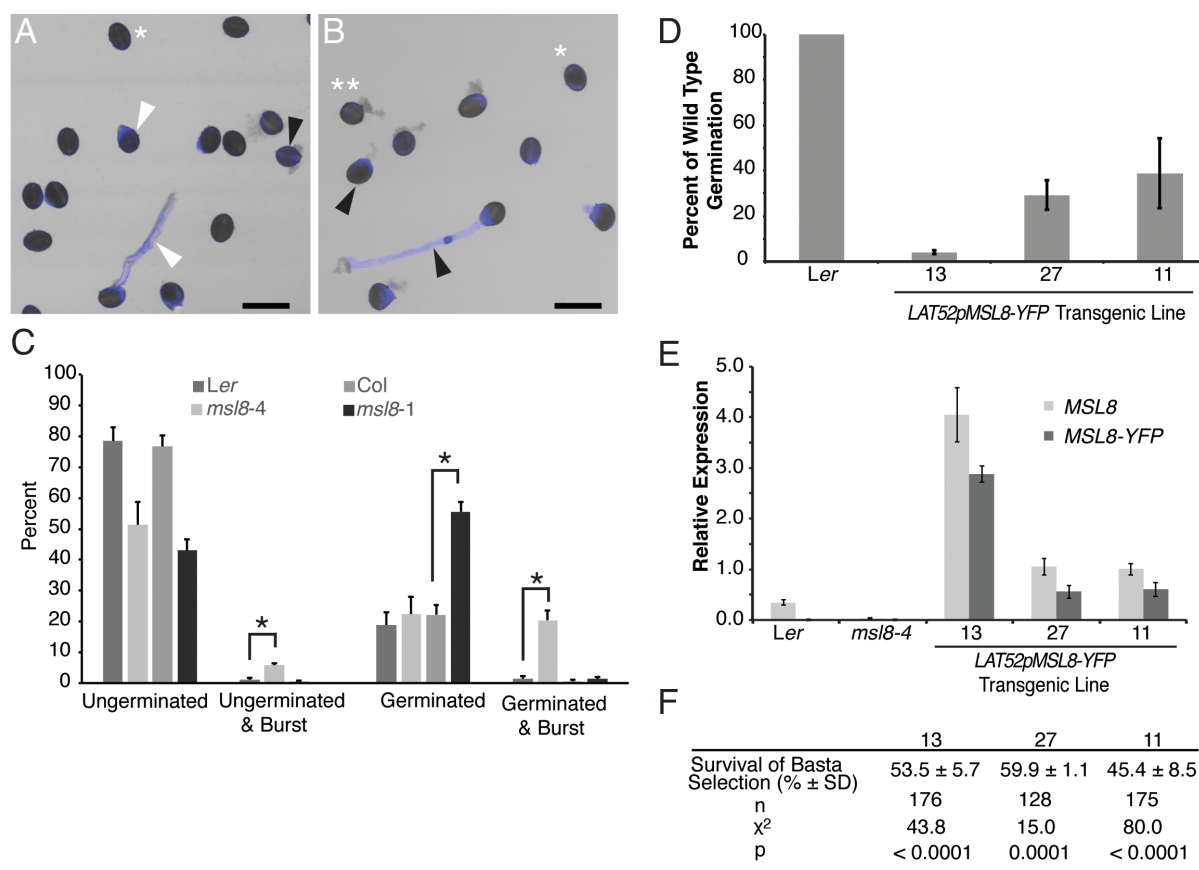
Fig. 4. MSL8 negatively regulates germination and is required for cellular integrity during germination. Wild type (**A**) and *msl8-4* (**B**) pollen germinated for four hours in liquid germination media and stained with aniline blue for callose, a marker of germination. Examples of ungerminated (single asterisk), ungerminated and burst (double asterisk), germinated (white arrow), or germinated and burst (black arrow) pollen are indicated. Scale bar is 50 μm. (**C**) Germination rate and bursting frequency in the indicated genotypes. N ≥ 396 per genotype. Asterisks indicate significant (p < 0.05) differences by Student's *t*-test (**D**) Percent germination overnight on solid media of pollen from wild type and three independent homozygous *LAT52pMSL8-YFP* transgenic lines. Bars indicate standard error. (**E**) Quantitative reverse-transcription PCR of *MSL8-YFP* and *MSL8* transcripts relative to *ACTIN* in flowers from L*er*, *msl8-4* and the *LAT52pMSL8-YFP* lines in (**D**). Two technical replicates of three biological replicates are presented. Error bars represent standard error. (**F**) Survival of selection for the Bialophos resistance gene in offspring of the *LAT52pMSL8-YFP* lines in (**D**). P-values were determined by a chi-squared test against 75% expected survival.

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251   **Supplementary Materials:**
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