The Mechanosensitive Ion Channel MSL10 Modulates Susceptibility to *Pseudomonas syringae* in *Arabidopsis thaliana*

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Plants sense and respond to molecular signals associated with the presence of pathogens and their virulence factors. Mechanical signals generated during pathogenic invasion may also be important, but their contributions have rarely been studied. Here, we investigate the potential role of a mechanosensitive ion channel, MscS-like (MSL)10, in defense against the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis thaliana*. We previously showed that overexpression of MSL10-GFP, phospho-mimetic versions of MSL10, and the gain-of-function allele *msl10-3G* all produce dwarfing, spontaneous cell death, and the hyperaccumulation of reactive oxygen species. These phenotypes are shared by many autoimmune mutants and are frequently suppressed by growth at high temperature in those lines. We found that the same was true for all three *MSL10* hypermorphs. In addition, we show that the SGT1/RAR1/HSP90 cochaperone complex was required for dwarfing and ectopic cell death, PAD4 and SID2 were partially required, and the immune regulators EDS1 and NDR1 were dispensable. All *MSL10* hypermorphs exhibited reduced susceptibility to infection by *P. syringae* strain Pto DC3000 and Pto DC3000 expressing the avirulence genes *avrRpt2* or *avrRpm1* but not Pto DC3000 *hrpL* and showed an accelerated induction of *PR1* expression compared with wild-type plants. Null *msl10-1* mutants were delayed in *PR1* induction and displayed modest susceptibility to infection by coronatine-deficient *P. syringae* pv. *tomato*. Finally, stomatal closure was reduced in *msl10-1* loss-of-function mutants in response to *P. syringae* pv. *tomato* COR⁺. These data show that MSL10 modulates pathogen responses and begin to address the possibility that mechanical signals are exploited by the plant for pathogen perception.

Keywords: *Arabidopsis thaliana*, mechanosensitive ion channel, MSL10, *Pseudomonas syringae*

Plant immune receptors achieve a two-tiered response to pathogens (Chiang and Coaker 2015; Jones et al. 2016; Zhou and Zhang 2020). The first line of defense relies on plasma membrane-localized pattern recognition receptors (PRRs), which facilitate molecular recognition of microbe- or damage-associated molecular patterns (MAMPs or DAMPs, respectively) and subsequently trigger pattern-triggered immunity (PTI) (DeFalco and Zipfel 2021; Saijo et al. 2018). However, pathogens evade PTI responses by delivering molecular virulence factors or effectors that subvert basal defenses into host cells (Toruño et al. 2016; Saur et al. 2021).

To counter such pathogens, plants have, in turn, evolved cytosolic receptors that constitute the second layer of plant defense (Jones et al. 2016). These intracellular receptors contain nucleotide-binding leucine-rich repeats and are thus known as NB-LRRs or NLRs. Both toll interleukin 1 (TIR)-NLR and coiled coil (CC)-NLRs detect the presence of intracellular effectors and activate effector-triggered immunity (ETI) (Cui et al. 2015). Some NLRs recognize and bind effectors directly, while others recognize the host proteins that effectors modify (Khan et al. 2018). It has recently been discovered that ZAR1 CC-NLRs oligomerize to form a large complex that serves to detect the activity of several bacterial effectors and is capable of channeling calcium and triggering defense responses (Bi et al. 2021; Wang et al. 2019). The TIR-NLRs ROQ1 and RPP1 also oligomerize to form “resistosomes” with nicotinamide dinucleoside hydrolase activity (Ma et al. 2020; Martin et al. 2020).

As described above, the molecular perception of pathogens and their effectors by PRRs and NLRs is central to the plant immune response. However, there is growing evidence that mechanical signals generated during invasion also elicit plant defense responses. For example, *Arabidopsis*, strawberry, and bean plants acquire transient immunity to necrotic or wounding pathogens following mild mechanical stimulus (Coutand 2020). It is not yet known if pathogens create detectable mechanical perturbations, if the mechanoperception of pathogens utilizes the same pathways as defenses do, nor how such signals would be detected.

One mechanism by which the plant may recognize pathogen-associated mechanical signals is the activation of mechanosensitive (MS) ion channels. Multiple families of MS ion channels mediate the flux of ions across a membrane in response to increased membrane tension in plants (Basu and Haswell 2017; Guerringue et al. 2018). Genetic and biochemical data support roles for several proteins that are homologs of known MS ion channels in the response to pathogens. Plants lacking the AtPIEZO1 channel are unable to fully suppress long-distance movement of viruses (Zhang et al. 2019), and OSCA1.3 is required for immunity-based stomatal

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closure (Thor et al. 2020). MCA family members are also implicated in pathogen signaling and feedback loops (Engelsdorf et al. 2018). The putative MS ion channel MscS-like (MSL) 4 contributes to basal immunity and MAMP-induced responses (Zhang et al. 2017).

MSL10 is a ubiquitously expressed homolog of MSL4 and is known to form a mechanically activated ion channel both in root cells and in *Xenopus* oocytes (Haswell et al. 2008; Maksaev and Haswell 2012), making it an intriguing candidate for a sensor of pathogen invasion. It is directly activated through membrane tension and is largely nonselective, with a slight preference for anions (Maksaev and Haswell 2012). Based on homology to other MSL channels, the MSL10 channel is likely a homoheptamer (Bass et al. 2002; Deng et al. 2020), though it may form multimeric complexes with its closest homolog, MSL9 (Haswell et al. 2008).

Plants constitutively overexpressing MSL10-GFP (green fluorescent protein) display severe growth retardation, hyperaccumulation of H₂O₂, induction of genes associated with stress response and ectopic cell death (Basu et al. 2020; Veley et al. 2014). These phenotypes are reproduced in plants harboring an ethylmethane sulphonate-induced gain-of-function point mutation in the soluble C terminus (S640L; *msl10-3G*) and in transgenic lines expressing an untagged version of *MSL10* containing seven Ser/Thr-to-Ala mutations in the soluble N terminus from the native (or genomic) context (*MSL10g^{7A}*) (Basu et al. 2020). Plants expressing wild-type (WT) *MSL10* (*MSL10g*) or a phospho-mimetic version of *MSL10* (*MSL10g^{7D}*), do not share these phenotypes, suggesting that dephosphorylation of N-terminal Ser/Thr residues leads to channel activation (Basu et al. 2020). Because MSL10-GFP overexpressors, *msl10-3G* mutants, and plants expressing *MSL10g^{7A}* all show the same gain-of-function phenotypes, we collectively refer to them here as *MSL10* hypermorphs. While the molecular mechanism by which *MSL10* hypermorphs are dysregulated has not yet been established, we have speculated that phosphorylation of the seven Ser/Thr residues in the MSL10 N terminus keeps it in an inactive signaling state and that either preventing phosphorylation with Ser/Thr-to-Ala mutations or overexpression of the GFP-tagged protein circumvents this regulation (Basu et al. 2020; Veley et al. 2014). We also recently proposed that an interaction between the soluble N and C termini of MSL10 monomers accompanies MSL10 activation and that the *msl10-3G* point mutation promotes or recapitulates this activation (Basu et al. 2020).

Dwarfism and spontaneous lesions are also observed in two previously characterized, partially overlapping classes of *Arabidopsis thaliana* mutants. Autoimmune mutants are unable to properly suppress immune responses in the absence of pathogens (van Wersch et al. 2016), while lesion mimic mutants (LMMs) exhibit uncontrolled programmed cell death on the leaf surface (Bruggeman et al. 2015). Prompted by these similarities between autoimmune and LMMs and *MSL10* hypermorphs, our previous characterizations of MSL10 as a sensor of membrane tension, and by the intriguing possibility that plants could sense mechanical signals of pathogen invasion, we investigated the potential role of MSL10 in plant defense responses against *Pseudomonas syringae*.

RESULTS

Growth at high temperatures suppresses the phenotypes associated with overexpression of MSL10-GFP, expression of *MSL10g^{7A}*, and the *msl10-3G* mutant.

Growth at elevated temperatures (28 to 30°C) can suppress the dwarfing and the localized ectopic cell death in autoimmune mutants (Hammoudi et al. 2018; Hua 2013; Huot et al. 2017), and we speculated that the same might be true for *MSL10*

hypermorphs. As shown previously (Veley et al. 2014), MSL10-GFP overexpressors were dramatically smaller than the WT when grown at 21°C (Fig. 1A and B). However, when grown at high temperature, MSL10-GFP overexpressing lines were nearly indistinguishable from WT, *msl10-1*, or *msl9-1 msl10-1* mutant plants in terms of rosette size and fresh weight. Elevated temperature did not substantially alter the level of transcripts from the endogenous *MSL10* gene in WT plants, *msl10-1* null mutants, or MSL10-GFP overexpression lines (Fig. 1C), and MSL10-GFP protein abundance was unaffected (Fig. 1D). Furthermore, MSL10-GFP was localized at the periphery of the cell at both normal and high temperatures in all three transgenic lines (Fig. 1E). Growth at high temperature also suppressed dwarfing in *msl10-3G* mutants and in plants expressing the *MSL10g^{7A}* transgene but did not affect the growth of plants expressing *MSL10g* or *MSL10g^{7D}*. High temperature did not appreciably affect transcript levels of endogenous or transgenic *MSL10* in these lines (Supplementary Fig. S1). Thus, growth at elevated temperatures relieved dwarfing in all three *MSL10* hypermorphs but did not do so by altering transcript levels, protein abundance, or subcellular localization. Growth at elevated temperature also suppressed previously documented cellular phenotypes of *MSL10* hypermorphs, including the hyperaccumulation of reactive oxygen species (ROS), ectopic cell death, and increased transcript levels of four genes previously established as hallmarks of MSL10 activity (Veley et al. 2014).

We assessed these phenotypes in rosette leaves from three-week-old WT and *msl10-1* and *msl10-3G* mutant plants, three independent transgenic lines overexpressing MSL10-GFP (previously described by Veley et al. [2014]), and *msl10-1* plants harboring *MSL10g*, *MSL10g^{7A}*, or *MSL10g^{7D}* transgenes (which contain a genomic copy of WT *MSL10*, *MSL10* with seven phospho-dead mutations in the soluble N-terminus, or *MSL10* with seven phospho-mimetic mutations in the soluble N terminus, respectively, all previously described by Basu et al. [2020]). When plants were grown at high temperatures, the increased O₂⁻, H₂O₂, and ectopic cell death observed in MSL10-GFP overexpression lines, the *msl10-3G* mutant, and *MSL10g^{7A}* transgenic lines were similar to that found in the WT (Fig. 2A, B, and C). *MSL10g* and *MSL10g^{7D}* lines and *msl10-1* mutants were indistinguishable from the WT when grown at high or at normal temperatures. The difference in scale between the data shown in Figure 2B and C can be attributed to different instruments used in analyzing ROS and cell death (discussed below). Finally, the transcript levels of four genes previously identified as reporters of MSL10 overactivation (Basu et al. 2020; Veley et al. 2014) were reduced to almost WT levels when MSL10-GFP overexpressing plants were grown at high temperatures (Fig. 2D). Taken together, these data show that growth at high temperature relieves all the phenotypes associated with the presence of hypermorphic alleles of *MSL10*. Because similar phenotypes in autoimmune mutants are also suppressed by high temperature (Hua 2013), these results suggested that the *MSL10* hypermorph phenotypes might also be derived from the overactivation of immune response pathways.

MSL10-GFP overexpression phenotypes require *SGT1*, *RAR1*, and *HSP90*.

To test the hypothesis that *MSL10* hypermorphs produce an autoimmune response, we asked if key genes involved in pathogen response were required for MSL10-GFP overexpression phenotypes. Most NLRs require *SUPPRESSOR of G2 ALLELE of SKP1* (*SGT1*) for function (Kadota et al. 2010). SGT1 is part of a cochaperone complex that includes REQUIRED for MLA12 RESISTANCE 1 (*RAR1*), and HEAT SHOCK PROTEIN 90 kD (*HSP90*) (Liu et al. 2004; Siligardi et al. 2018; Takahashi et al. 2003). SGT1, RAR1, and HSP90 interact directly with NLRs

and are required for their accumulation and subcellular trafficking (Borrelli et al. 2018). Thus, in the absence of any known NLR associated with MSL10-induced downstream autoimmune response or any evidence of MSL10 acting as a target for a pathogen effector protein, we tested if silencing of *SGT1*, *RAR1*, and *HSP90* by virus-induced gene silencing (VIGS) (Baulcombe 1999) could abolish the cell death triggered by transiently overexpressed MSL10-GFP in *Nicotiana benthamiana*.

Leaves were agroinfiltrated with VIGS constructs (*TRV2:SGT1*, *TRV2:RAR1*, *TRV2:HSP90*, or empty vector). After 10 days, transcript levels of each target gene were reduced by approximately 80% (Supplementary Fig. S2A). After another 10 days, leaves were subjected to a second agroinfiltration with *35S::MSL10-GFP*, *35S::MSL10^{7D}-GFP*, or *35S::MSL10^{7A}-GFP*. Cell death was quantified 5 days later, using dual fluorescein diacetate (FDA) propidium iodide (PI) staining as described by Veley et al. (2014). As

Fig. 1. Growth at high temperatures suppresses dwarfing in MSL10-GFP (green fluorescent protein) overexpressors. **A**, Three-week-old seedlings from wild type (WT), *msl10-1*, and three independent T4 homozygous lines overexpressing MSL10-GFP, grown side-by-side on soil under 24 h of light at 21 or 30°C. Bars = 0.5 cm. **B**, Fresh weight (FW) of the aerial portion of plants grown as in A. Error bars indicate standard deviation of the mean FW from 30 plants, grown in three independent trials, 10 plants per trial. **C**, *MSL10* transcript abundance relative to WT in WT, *msl10-1*, or *35S::MSL10-GFP-15* plants, grown as in A. Inset, WT and *msl10-1* lines only. Expression levels were normalized to *EF1α* and *UBQ5*. Error bars indicate standard error of the mean from three independent trials, three technical replicates per trial. **D**, Immunoblot of protein extracts from rosette leaves of two-week-old plants. MSL10-GFP was detected with an anti-GFP primary antibody (top), then, the blot was stripped and reprobed with an anti-α tubulin primary antibody (bottom). The intensity of each MSL10-GFP protein band, normalized to tubulin, is indicated at the bottom of the blot. **E**, Confocal images of leaf epidermal cells from plants grown as in A. GFP signal is shown in green. All images were obtained using identical settings. Bars = 20 μm. In B and C, different letters indicate significant differences, as determined by two-way analysis of variance followed by Tukey's post hoc test ($P < 0.05$).

Fig. 2. Growth at high temperatures suppresses multiple cellular and molecular phenotypes associated with *MSL10* hypermorphs. **A**, Colorimetric quantification of superoxide content, as indicated by nitroblue tetrazolium chloride (NBT)–formazan deposition, in rosette leaves in three-week-old plants of the indicated genotypes grown at 21 or 28°C under 24 h of light. **B**, Hydrogen peroxide (H_2O_2) content in rosette leaves grown as in A, quantified with the Amplex Red-coupled quantitative assay. **C**, Ectopic cell death indicated by trypan blue staining in rosette leaves grown as in A. **D**, Relative transcript abundance of four previously characterized stress-responsive genes. In all panels, different letters indicate significant differences, as determined by two-way analysis of variance followed by Tukey's post hoc test ($P < 0.05$). A to C present the means $\pm$ standard deviation from three independent trials, five to ten leaves per genotype.

expected, in leaves that had been pre-infiltrated with an empty VIGS vector, transient expression of WT MSL10-GFP resulted in the death of approximately 27% of *N. benthamiana* epidermal cells and expression of MSL10^{7A}-GFP triggered even more cell death (approximately 39%), while expression of MSL10^{7D}-GFP resulted in the death of just 11% of cells (Fig. 3A). However, we found that

silencing of either *SGT1*, *RAR1*, or *HSP90* almost completely prevented cell death in leaves that were transiently expressing MSL10-GFP (approximately 10%) or MSL10^{7A}-GFP (approximately 16%). Immunoblotting showed MSL10-GFP protein levels were not significantly different in silenced and non-silenced samples (Supplementary Fig. S2B to D; Supplementary Table S2). Rather,

Fig. 3. *SGT1b*, *RAR1*, and *HSP90* are required for phenotypes associated with MSL10-GFP overexpression in tobacco and *Arabidopsis*. **A**, Quantification of cell death, as determined by dual staining fluorescein diacetate and propidium iodide in epidermal cells from *Nicotiana benthamiana* leaves transiently overexpressing MSL10-GFP and its phospho-variants when *SGT1b*, *RAR1*, or *HSP90* was silenced. The means and standard deviation (SD) from three independent trials are shown. Each datapoint represents the percent cell death from approximately 100 epidermal cells from a single leaf. For each genotype, three leaves each from three different plants were assessed per trial, for a total of three independent trials. **B**, 35S:MSL10-GFP seedlings segregating *sgt1b-1*, *rar1-21*, or *hsp90.1-1*, grown side-by-side on soil under 24 h of light at 21°C. Left panel bars = 0.5 cm; right panel bars = 0.35 cm. **C**, Immunoblot showing MSL10-GFP levels in total protein extracted from three-week-old rosette leaves grown as in B. Relative MSL10-GFP levels (normalized to tubulin) are shown at the bottom of each lane; replicates and statistics are shown in Supplementary Table S2. **D**, Hydrogen peroxide (left) and trypan blue (right) content in rosette leaves. The means and SD from three independent trials, each with three leaves are presented. In A and D, different letters indicate significant differences, as determined by two-way analysis of variance followed by Tukey's post hoc test (P < 0.05).

they suggest that *SGT1*, *RAR1*, or *HSP90* function downstream of *MSL10* and are required for its ability to induce programmed cell death.

We next investigated the same question in *Arabidopsis* plants stably expressing *35S::MSL10-GFP* into the T-DNA insertion lines *sgt1b-1* (SALK_026606) and *hsp90.1-1* (SALK_007614; (Takahashi et al. 2003) and the point mutant *rar1-21* (Tornero et al. 2002). The *sgt1b-1* mutant line has a T-DNA inserted in the promoter region of the *SGT1b* gene and *SGT1b* expression was reduced to approximately 60% of that in WT plants (Supplementary Fig. S3A and B), indicating that the *sgt1b-1* mutant is a knock-down allele of *SGT1b*. The *sgt1b-1*, *rar1-21*, and *hsp90.1* mutants did not exhibit detectable morphological differences compared with WT plants (Supplementary Fig. S3C). To avoid variability in transgene expression due to position effect, we decided to first incorporate a transgene in a mutant background and cross the T1 selected plant with WT Col-0. The segregating F2 progenies of homozygous knock-out mutant and WT (both harboring the transgene integrated in the same location in the chromosome of either genetic background) were considered for further analysis.

As expected, plants expressing *35S::MSL10-GFP* in the WT *SGT1b*, *RAR1*, and *HSP90.1* backgrounds exhibited dwarfing and ectopic cell death, as assessed by the appearance of yellowish-brown lesions on rosette leaves. (Fig. 3B). However, these phenotypes were largely absent in *MSL10-GFP* overexpressing siblings homozygous for *sgt1b-1*, *rar1-21*, and *hsp90.1-1*. Immunoblotting and confocal imaging showed that this loss of cell death could not be attributed to a decrease in *MSL10-GFP* protein abundance (Fig. 3C; Supplementary Fig. S3D; Supplementary Table S2). H_2O_2 hyperaccumulation and ectopic cell death were also markedly reduced in *sgt1b-1 35S::MSL10-GFP* (approximately 50 and 65%), *rar1-21 35S::MSL10-GFP* (approximately 60 and 70%), and *hsp90.1-1 35S::MSL10-GFP* (approximately 50 and 67%) lines compared with their *35S::MSL10-GFP* siblings (Fig. 3D). We obtained similar results with a recently described null allele of *SGT1b* (*sgt1b-2*, GABI_857A04) (Zhang et al. 2015) (Supplementary Fig. S3E, F, and G). These results collectively indicate that the dwarfing, H_2O_2 accumulation, and ectopic cell death associated with *MSL10-GFP* overexpression require the surveillance complex SGT1/RAR1/HSP90.1 in both *N. benthamiana* and *Arabidopsis*. However, this effect does not appear to be mediated through protein-protein interactions, as *SGT1b*, *RAR1*, and *HSP90.1* did not associate with *MSL10* in bimolecular fluorescence complementation (BiFC) assays (Supplementary Fig. S4). These data suggest that *MSL10* indirectly requires the function of the surveillance complex to induce programmed cell death, likely through converging pathways. However, we cannot rule out the possibility that a combination or even all three of these proteins must be present for *MSL10* to interact stably with the SGT1/RAR1/HSP90.1 complex.

The phenotypes associated with *MSL10-GFP* overexpression are independent of *EDS1* and *NDR1*.

Downstream of NLR function, the immune regulator ENHANCED DISEASE SUSCEPTIBILITY (*EDS1*) is a lipase-like protein that serves to connect TIR-NLR signaling with downstream events, including programmed cell death, production of the defense hormone salicylic acid (SA), and defense gene expression (Dongus and Parker 2021). NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (*NDR1*), a membrane-associated integrin-like protein, plays a similar role for CC-NLRs (Century et al. 1995, 1997; Coppinger et al. 2004; Knepper et al. 2011). To investigate whether disruption of *EDS1* or *NDR1* are required for dwarfing or cell death observed in *MSL10-GFP* overexpression lines or the *msl10-3G* background, we introduced *35S::MSL10-GFP* into the *eds1-23* (SALK_057149, (Song 2016) and the *ndr1-1* (Century

et al. 1997) null mutant backgrounds by transformation. As anticipated, expression of *35S::MSL10-GFP* in WT plants resulted in severe dwarfing and the appearance of yellowish-brown lesions on rosette leaves (Fig. 4A, top panels) and trypan blue-stained patches on leaves (Fig. 4A, bottom panels) compared with the WT or to untransformed *eds1-23* or *ndr1-1* mutants. We found that neither the *eds1-23* nor the *ndr1-1* mutant background appreciably affected the phenotypic results of *MSL10-GFP* overexpression, and immunoblotting confirmed similar *MSL10-GFP* levels in all lines (Fig. 4B). Similarly, neither the *eds1-23* nor the *ndr1-1* alleles altered dwarfing or ectopic cell death observed in the *msl10-3G* background (Fig. 4C). Collectively, these results indicate that the phenotypes associated with *MSL10* hypermorphs are independent of *EDS1* and *NDR1*.

PAD4 and *SID2* are partially required for the dwarfing and ectopic cell death associated with *MSL10-GFP* overexpression.

SA accumulation is often associated with inhibition of growth, spontaneous cell death, and immune responses (Bruggeman et al. 2015; Peng et al. 2021). This prompted us to investigate whether SA biosynthesis might be required for *MSL10*-associated phenotypes. Toward this goal, we crossed plants expressing *35S::MSL10-GFP* with *PHYTOALEXIN DEFICIENT 4-1* (*pad4-1*), *SA INDUCTION DEFICIENT2-2* (*sid2-2*) double mutants (Jirage et al. 1999; Wildermuth et al. 2001). The *SID2* gene encodes ICS1, a key SA biosynthetic enzyme, while *PAD4* is a lipase-like protein that is involved in transcriptional upregulation of SA accumulation (Ng et al. 2011). We followed the approach described above to assess transgenic siblings with different genetic backgrounds. Four-week-old *pad4-1sid2-2* siblings were phenotypically indistinguishable from the WT (*PAD4 SID2*) parental line, but *pad4-1sid2-2* siblings overexpressing *MSL10-GFP* displayed partial suppression of the dwarfing and fresh weight seen in the *35S::MSL10-GFP* line (Fig. 5A and B). Consistent with these phenotypic features, the level of ectopic cell death in *pad4-1sid2-2 35S::MSL10-GFP* leaves was approximately 50% lower than that observed in *35S::MSL10-GFP* overexpression lines (Fig. 5C). *MSL10-GFP* levels were comparable in both types of siblings, as assessed by immunoblotting (Fig. 5D; Supplementary Table S2). These results suggest that SA biosynthesis influences but is not required for the autoimmune phenotypes associated with *MSL10-GFP* overexpression.

Hypermorph alleles of *MSL10* confer reduced susceptibility to *Pseudomonas syringae*.

Pseudomonas syringae is a model for plant-pathogen studies, and programmed cell death is commonly triggered during the hypersensitive response of *Arabidopsis* to *Pseudomonas syringae* interactions (Xin and He 2013). Since *MSL10* hypermorphs are known to trigger cell death in response to cell swelling (Basu and Haswell 2020) and cell death is a typical immune response, we asked whether *MSL10* contributes to pathogen resistance. Plants were grown at 28°C under short-day conditions for five to six weeks to suppress the cell death and dwarfing phenotypes of *MSL10* hypermorphs prior to infection. Then, since elevated temperature suppresses both plant immunity and pathogen virulence (Huot et al. 2017), five- to six-week-old plants were transferred to 21°C 24 h before inoculation with bacterial cultures. Using this strategy (Supplementary Fig. S5), the levels of H_2O_2 and cell-death plants harboring *MSL10* hypermorph alleles remained indistinguishable from the WT plants at the time of infection, 1 day after transfer to 21°C.

Fully expanded leaves were syringe-inoculated with *Pto* DC3000, which is virulent and can multiply to high levels (100- to 1,000-fold) in WT Col-0 plants. At the start of the experiment, no differences in bacterial counts were observed among any lines (Fig. 6A). At 3 days postinfection, *msl10-3G* mutants supported

significantly lower bacterial growth than WT plants, as did plants overexpressing MSL10-GFP and plants expressing MSL10g^{7A}. In contrast, phospho-mimetic versions of *MSL10* transgenes (35S::*MSL10-GFP*^{4D} or *MSL10g*^{7D}) and the *msl10-1* mutant had no apparent effect relative to the WT. The *pad4-1sid2-2* mutant, which does not accumulate SA in response to infection, exhibited enhanced susceptibility to *Pto* DC3000.

The enhanced resistance against *Pto* DC3000 exhibited in lines expressing *MSL10* hypermorphs suggested that MSL10

may be involved in basal plant defense responses. To test this idea, we syringe-inoculated plants with a mutant strain of *Pto* DC3000, *hrpL*. This mutant strain lacks the HrpL transcription factor that controls the expression of type III secretion system genes required for effector secretion, cannot suppress basal defense, and therefore, does not grow to high levels in WT Col-0 plants (Zwiesler-Vollick et al. 2002). When inoculated with *Pto* DC3000 *hrpL* mutant bacteria, none of the lines showed apparent differences in viable bacterial counts (Fig. 6B). In addition,

Fig. 4. Disruption of *EDS1* or *NDR1* fails to suppress phenotypes associated with *MSL10* hypermorphs. **A**, Top row, five-week-old T1 plants grown side-by-side in 24 h of light at 21°C. Bars = 2 cm. Bottom row, rosette leaves from the same plants stained with trypan blue to visualize dead cells. Bars = 200 µm. **B**, Immunoblot of protein extracts from leaf tissue of T1 lines. Relative MSL10-GFP levels (normalized to tubulin) are shown at the bottom of each lane; replicates and statistics are shown in Supplementary Table S2. **C**, *msl10-3G* siblings segregating *eds1-23* or *ndr1-1* alleles. Top row, four-week-old plants grown as in A. Bars = 2 cm. Bottom row, dead cells in rosette leaves visualized by trypan blue staining. Bars = 200 µm.

we tested the resistance of these lines to avirulent *Pseudomonas syringae* expressing two well-characterized effectors, *Pto* DC3000 *avrRpt2* and *Pto* DC3000 *avrRpm1* (Mackey et al. 2003, 2002). We found that *MSL10*-GFP overexpression lines exhibited enhanced resistance to *Pto avrRpt2* (Fig. 6C) and all three hypermorphs exhibited enhanced resistance to *Pto avrRpm1* (Fig. 6D), as compared with WT plants. However, *msl10-1* mutants and *MSL10g^{7D}* lines were indistinguishable from the WT in these assays. In summary, overactive *MSL10* alleles conferred reduced susceptibility to *Pto* DC3000, *Pto avrRpt2*, and *Pto avrRpm1*, but not to *Pto* DC3000 *hrpL*. Taken together, these data suggest that *MSL10* hypermorphs may act as an autoimmune mutant.

***MSL10* is required for normal PATHOGENESIS-RELATED 1 (*PR1*) induction in response to *Pseudomonas* infection.**

The ectopic cell death observed in autoimmune mutants is often accompanied by constitutive upregulation of defense-related genes like *PR1* (Li et al. 2010; Wang et al. 2017; Xu et al. 2015). To investigate whether the same is true of *MSL10* hypermorphs, a time-course experiment was performed to evaluate *PR1* transcript accumulation in response to *Pto* DC3000 infection. We found that basal levels of *PR1* transcripts were the same in all lines tested. However, the kinetics of induction was different. In WT plants, increased levels of *PR1* transcripts were detected by 6 h and continued to increase at the 12- and 24-h timepoints (Fig. 7A). *PR1* transcript levels also increased after infection in *msl10-1* mutants, but the increase was not observable until 12 h after treatment. On the other hand, *PR1* expression in the *msl10-3G* mutant (approximately 18 to 45-fold) and in *MSL10*-GFP overexpression lines (approximately 24 to 57-fold)

was higher than the WT (approximately five- to 15-fold) as early as 6 h after *Pto* DC3000 infection. At all timepoints tested, plants expressing phospho-mimetic 35S::*MSL10^{4D}*-GFP and *MSL10g* complemented lines displayed similar *PR1* expression kinetics as WT plants. We did observe small increases in *PR1* expression at later timepoints in all genotypes in response to mock infection. This may be attributed to mechanical or osmotic stress resulting from syringe-infiltration (Chandrashekar et al. 2018). Thus, *MSL10* positively influences *PR1* induction after *Pto* DC3000 infection.

***MSL10* is required for normal basal defense, rapid *PR1* induction, and full stomatal closure in response to *Pto* DC3000 coronatine-deficient (*COR⁻*) mutant.**

The observation that *msl10-1* mutants showed a delayed and attenuated induction of *PR1* led us to investigate the role of *MSL10* in responding to *Pto* infection under sensitized conditions. Under natural conditions, the *Pseudomonas* pathogen enters plants either through wounds or natural openings like stomata and hydathodes (Melotto et al. 2008). Syringe-infiltration directly delivers the bacteria into the apoplast, bypassing these natural entry mechanisms. To assess bacterial growth under more realistic conditions, we spray-inoculated plants with *Pto* DC3000. As with syringe-infiltration, *msl10-3G* mutants displayed heightened resistance to *Pto* DC3000 compared with WT plants, while *msl10-1* mutants and the *MSL10g* line were indistinguishable from the WT. Coronatine-deficient (*COR⁻*) bacterial mutants have severely compromised virulence when inoculated onto the leaf surface and are useful to characterize mutants with slightly weakened defense responses (Geng et al. 2014). When plants were spray-inoculated with a *Pto* DC3000 *COR⁻* mutant (Brooks et al. 2004), *msl10-3G* mutants again displayed enhanced resistance compared with the WT at 2 or 3 days after infection (Fig. 7B). Importantly, when using this weaker pathogen and spray-inoculation, *msl10-1* showed reduced resistance compared with WT plants at both timepoints. We also observed a similar profile of *PR1* induction in response to infection with the *Pto* DC3000 *COR⁻* mutant strain. *MSL10* hypermorphs exhibited higher levels of *PR1* transcripts, and *msl10-1* mutants displayed a delayed and reduced induction of *PR1* transcripts (Fig. 7C). These results indicate that *MSL10* is required for normal levels of basal defense or innate immunity when presented with modest pathogenic challenges.

As *Pto COR⁻* strains are unable to inhibit stomatal closure and early PTI-mediated basal defense responses (Melotto et al. 2006), we hypothesized that the increased susceptibility in *msl10-1* mutants might be related to defects in stomatal closure. To test this hypothesis, we measured stomatal aperture from the leaf epidermis of WT, *msl10-1*, *MSL10g*, and *msl10-3G* lines exposed to the *Pto* DC3000 *COR⁻* mutant. WT leaves sprayed with *Pto* DC3000 briefly closed, then reopened, their stomates (Fig. 7D, left), while the stomates of WT leaves spray-inoculated with the *Pto* DC3000 *COR⁻* mutant closed and did not open again (Fig. 7D, right). All genotypes tested behaved similarly when spray-inoculated with DC3000. However, in response to the *Pto* DC3000 *COR⁻* mutant, the stomates of *msl10-1* mutant were significantly more open than all the other genotypes both at 1 and at 4 h after infection. To determine the specificity of this defect, plants were treated with abscisic acid (ABA), which is known to induce rapid closure (Kim et al. 2010). ABA treatment efficiently produced stomatal closing in all genotypes after 2 h of ABA incubation, establishing that there is no generic defect in the ability of *msl10-1* stomates to close (Fig. 7E). Taken together, these results suggest that *MSL10* is required for a robust and rapid response to pathogen invasion and its absence leads to delayed signaling, slower stomatal closure, and increased susceptibility.

Fig. 5. *PAD4* and *SID2* are partially required for the phenotypes associated with overexpression of *MSL10*-GFP. **A, Four-week-old plants grown side-by-side in 24 h of light at 21°C. **B**, Average fresh weight (FW) of rosettes grown as in **A**. **C**, Ectopic cell death indicated by trypan blue staining in rosette leaves. **D**, Immunoblot of *MSL10*-GFP protein levels from three-week old rosette leaves grown as in **A**. Relative *MSL10*-GFP levels (normalized to RuBisCO) are shown at the bottom of each lane; replicates and statistics are shown in Supplementary Table S2. In **B** and **C**, the mean and standard deviation of three independent trials, eight to ten leaves each, are presented, and different letters indicate significant differences, as determined by two-way analysis of variance followed by Tukey's post hoc test ($P < 0.05$).**

DISCUSSION

Considerable progress has been made in identifying and characterizing plant receptors involved in detecting bacterial effectors and MAMPs, but less is known about noncanonical players involved in plant defense. Here, we investigated the role of the mechanosensitive ion channel *MSL10* in plant immunity. We found that growth at an elevated temperature, loss of the *SGT1/RAR1/HSP90* chaperone complex, or disruption of SA biosynthesis fully or partially alleviated all the phenotypes triggered by

MSL10 hypermorphic alleles that we tested. These phenotypes, most of which are associated with enhanced basal defenses, included dwarfing, ectopic cell death, hyperaccumulation of ROS, and a characteristic gene expression profile (Figs. 1, 2, 3, and 5; Supplementary Figs. S1, S2, and S3). These results suggested that *MSL10* hypermorphs are similar to autoimmune mutants. However, the NLR signal transducers *EDS1* and *NDR1* were not required (Fig. 6). *MSL10* hypermorphs exhibited enhanced disease resistance against both virulent and avirulent strains of *Pseudomonas syringae* but not *hrpL* (Fig. 6), and *msl10*

Fig. 6. *MSL10* hypermorphs confer reduced susceptibility to *Pseudomonas syringae*. **A to D**, Bacterial growth in leaves from plants grown for 5 weeks side-by-side on soil at 28°C under a 10-h-light and 14-h-dark regime and then transferred to 21°C for 1 day before syringe inoculation. Means and standard deviation from three independent trials, each with six leaf extracts containing two leaf discs per genotype are presented. Different letters indicate a significant difference as determined by two-way analysis of variance followed by Tukey's post hoc test ($P < 0.05$).

null mutants showed increased susceptibility to infection by *COR⁻ Pto* (Fig. 7). We also found that *MSL10* promotes precocious *PR1* induction and is required for normal stomatal closure in response to infection by a *Pto* DC3000 *COR⁻* mutant (Fig. 7).

***MSL10* hypermorphs compared with lesion mimic and autoimmune mutants.**

MSL10 hypermorphs strongly resemble a category of mutants called LMMs. LMMs display spontaneous cell death in the absence of any pathogenic infection, mechanical stress, or abiotic stress (Bruggeman et al. 2015; Moeder and Yoshioka 2008). Like *MSL10* hypermorphs, LMMs often share other phenotypes, including dwarfing and the constitutive activation of defense responses. Also similar to *MSL10* hypermorphs, the phenotypes of some LMMs are temperature-sensitive (Huang et al. 2010; Yang and Hua 2004; Yang et al. 2010). High temperatures suppress immune responses through multiple mechanisms, including resistance (*R*)-gene mediated resistance (Hua 2013) and SA production (Huot et al. 2017), and are known to affect MS ion channel activity (Ding and Pickard 1993). As a result, future work will be needed to determine the mechanism by which temperature affects the phenotypes of *MSL10* hypermorphs. Some LMMs are autoimmune mutants in that their phenotypes result from the loss of repression of pathogen responses (Chakraborty et al. 2018; van Wersch et al. 2016). Autoimmune effects can result from the overactivation of PRRs and NLR proteins, the loss of negative regulators of NLRs, and the disruption of NLR-guardee interactions and mis-regulation of SA biosynthesis, either singly or in combination. In this study, we provide evidence that the LMM-like phenotypes exhibited by *MSL10* hypermorphs are due to the induction of autoimmune responses (discussed below). However, unlike canonical and known autoimmune mutants, *MSL10* hypermorphs did not exhibit increased basal expression of the *PR1* gene (Fig. 7A) (Mauch-Mani et al. 2017).

***MSL10* and *R* gene-mediated immunity.**

The SGT1b/RAR1/HSP90 cochaperone complex is known to function as an integral component of ETI (van Wersch et al. 2020). Silencing of SGT1/RAR1/HSP90 components alleviates autoimmune responses in several autoimmune mutants. For example, SGT1b is a positive regulator of the autoimmune response displayed by *chs3* mutants (Xu et al. 2015), HSP90.3 is required for the constitutive activation of defense response in the *saull-1* mutant (Liang et al. 2020), and *topp4-1* autoimmune phenotypes require RAR1 and HSP90.2 (Yan et al. 2019). Similarly, the spontaneous cell death and hyperaccumulation of ROS exhibited by plants overexpressing *MSL10*-GFP required *SGT1b*, *RAR1*, and *HSP90* in both tobacco and *Arabidopsis* (Fig. 3; Supplementary Figs. S2 and S3).

Taken together, these data suggest that *MSL10* acts upstream of *R* gene-mediated immune responses. However, most NLR-mediated signaling requires either *EDS1* or *NDR1*, and we observed that disruption of *EDS1* and *NDR1* did not alleviate the autoimmune-like phenotypes associated with *MSL10* hypermorphs. Furthermore, while autoimmunity can be caused by defects in guarder proteins, *msl10* null mutants do not show an autoimmune response under unstressed conditions, indicating that *MSL10* is unlikely to function as a guarder. A few scenarios fit the data presented here. *MSL10* could function upstream of a pathway that involves *R* proteins that are *EDS1*- and *NDR1*-independent. There is some precedent for such a pathway; the effector Rip1E from *Ralstonia solanacearum* requires *SGT1* but not *EDS1* to trigger immunity in *N. benthamiana* (Sang et al. 2020). Alternatively, *MSL10* could function in a pathway that is entirely independent of *R* genes. For the latter to be the case, the SGT1 complex would have to be involved in an *R*-gene independent function as well, and both HSP90 and SGT1b have been shown

to interact with the receptors for plant hormones auxin and jasmonic acid (Wang et al. 2016; Zhang et al. 2015). In summary, there are several ways in which *MSL10*-based signals could impinge on known pathogen response pathways; additional experiments will be needed to distinguish them.

MS ion channels in pathogen perception.

Cation fluxes are immediate and essential parts of the host immune response (Moeder et al. 2019), and it was recently reported that the OSCA1 mechanosensitive cation channel is required for defense-induced stomatal closure (Thor et al. 2020). While *MSL10* is unlikely to transport calcium directly (Maksaev and Haswell 2012), the release of anions through *MSL10* could depolarize the plasma membrane, thereby indirectly activating calcium channels (Guerringue et al. 2018). Indeed, *MSL10* is closely associated with and required for the cytoplasmic calcium transient associated with cell swelling (Basu and Haswell 2020). However, the ability to trigger dwarfing and programmed cell death may be triggered independently of ion flux through *MSL10* (Maksaev et al. 2018; Veley et al. 2014). Thus, it is possible that *MSL10* serves as a sensor of membrane integrity that is triggered by changes in membrane tension resulting from *Pseudomonas* infection, either due to the insertion or assembly of the type III secretion apparatus within the plasma membrane or indirectly to events triggered by cytoplasmic effectors such as water-soaking (though water-soaking is not seen with avirulent strains [Xin et al. 2016]). Activation of *MSL10* could then lead to calcium transients or to nonconducting interactions with components of *R* gene-mediated immunity, eventually leading to one or both *PR1* induction and stomatal closure. Interestingly, the putative MS ion channel *MSL4*, a close homolog of *MSL10*, interacts with the defense regulator ACD6 and is required for PRR-mediated basal immunity responses (Zhang et al. 2017) and AtPIEZO1 is required for long distance virus translocation (Zhang et al. 2019). Taken together, these results suggest that MS ion channels play diverse, yet-to-be-discovered roles in plant defense.

MATERIALS AND METHODS

Plant lines and growth conditions.

Arabidopsis thaliana plants used in this study were of the Col-0 ecotype. The following mutants were used: *sid2-2* (Wildermuth et al. 2001), *pad4-1* (Jirage et al. 1999), *eds1-23* (SALK_057149 [Song 2016]), *rar1-21* (Tornero et al. 2002), *sgt1b-2* (GABI_857A04 [Zhang et al. 2015]), *sgt1b-1* (SALK_026606), *ndr1-1* (Century et al. 1997), *hsp90.1-1* (SALK_007614 [Takahashi et al. 2003]), *fls2* (SALK_141277 [Guo et al. 2014]), *msl10-1* (SALK_076254), *msl10-3G* (Basu et al. 2020; Zou et al. 2016), and *msl9-1* (SALK_114626 [Haswell et al. 2008]). In most experiments, plants were grown on soil at 21°C under a 24-h light regime (approximately 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$). For experiments done at elevated temperature, plants were grown on soil at high temperatures that ranged from 28 to 30°C with 24-h light. For bacterial infection assays and stomatal aperture measurements, plants were grown at 21°C under a 24-h light regime for 10 days, until the seedling germinated, followed by 28°C under short-day conditions (10 h of light and 14 h of dark) for 5 weeks. Plants were then acclimatized for 24 h at 21°C in short-day conditions before subjecting them to bacterial infection. The *MSL10*-GFP overexpression lines (lines 7-1, 12-3, and 15-2) and *MSL10g*, *MSL10g^{7A}*, and *MSL10g^{7D}* plant lines are described in earlier studies (Basu et al. 2020; Veley et al. 2014).

Generation of transgenic lines.

pENTR-*MSL10* (Haswell et al. 2008) was introduced into the pEarleyGate destination binary vector pEG103 (Earley et al. 2006) using LR clonase. The resulting 35S::*MSL10*-GFP construct was

Fig. 7. *MSL10* modulates the normal induction of *PR1*, susceptibility to the *Pto* DC3000 coronatine-deficient (*COR*⁻) mutant, and stomatal closure. **A**, *PR1* transcript abundance relative to the wild type (WT). RNA was isolated from plants grown and was syringe-inoculated with *Pto* DC3000. **B**, Bacterial growth in leaves after spray-inoculation with the *Pto* DC3000 *COR*⁻ mutant. The means and standard deviation (SD) from five independent trials, each with five to nine leaf extracts, are shown. Each extract contained two leaf discs. **C**, *PR1* transcript abundance relative to WT, performed as in **A**, except that plants were spray-inoculated with the *Pto* DC3000 *COR*⁻ mutant. **D** and **E**, Stomatal aperture measurements. Fully expanded, healthy leaves from 5-week-old plants were immersed in a suspension of *Pto* DC3000 or the *Pto* DC3000 *COR*⁻ mutant or 10 μ M abscisic acid (ABA), and stomatal apertures were measured at the indicated timepoints. Means and SD from three independent trials, each with 16 apertures, are shown. Different letters indicate a significant difference as determined by two-way analysis of variance followed by Tukey's post hoc test ($P < 0.05$). CFU = colony-forming units; mock = 10 mM MgCl₂.

transformed into Col-0 (WT), *sgt1b*, *rar1-21*, *hsp90.1-1*, *pad4-1sid2-2*, *eds1-23*, or *ndr1-1* mutant backgrounds by *Agrobacterium tumefaciens* (GV3101)-mediated floral dipping (Clough and Bent 1998). Transgenic plants were selected on soil by spraying with 200 mg of Basta per liter (Finale Herbicide, Southern States Farm Supply), followed by segregation analysis and selection of homozygous transgenic lines with single insertion of transgene on Murashige-Skoog plates supplemented with 20 mg of Basta per liter. Sequences of the primers used for genotyping transgenes or the mutant background are listed in Supplementary Table S1. Since transgene expression in a knock-out mutant background relative to the transgene expression in the WT plant does not involve identical genetic background, it is difficult to compare phenotypes among the transgenic plants. To eliminate such issues, we first introduced the transgene into the knock-out mutant line and then crossed the T1 transgenic line with the WT Col-0 line. Segregating homozygous F2 siblings with identical genetic backgrounds were used for comparison. Plants with the *msl10-3G* allele were crossed to *eds1-23* and *ndr1-1* plants. F2 progeny homozygous for the *msl10-3G* allele were identified by genotyping, and comparisons were made between those F2 siblings homozygous for WT or mutant *eds1-23* and *ndr1-1* alleles.

Gene expression analysis.

Quantitative reverse-transcription PCR (qRT-PCR) was performed as described previously (Basu et al. 2020), with minor modifications. In most experiments, total RNA was isolated from the rosette leaves (5th and 9th) of healthy 3-week-old plants using the RNeasy plant mini kit (Qiagen) including DNase I treatment. Rosette leaves from five-to-six-week-old plants were used for RNA extraction for transcript analysis in response to bacterial infection. Reverse transcription was performed using 2 µg of total RNA and oligo(dT)₂₀ primers with M-MLV Reverse Transcriptase (Promega). All results shown include data from three independent experiments; for each treatment three technical replicates were performed for each of three independent experiments. Expression levels were normalized to the geometric mean of two reference genes, *UBQ5* and *EF1α*. Finally, the relative abundance of transcripts was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Semiquantitative RT-PCR was performed as described by Velej et al. (2014). Sequences of the primers used are listed in Supplementary Table S1.

Quantification of ROS in tissue extracts.

H₂O₂ levels in leaf extracts were measured using the Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Invitrogen) (Basu et al. 2020). Three to five independent experiments were used for each condition at each time interval. The leaves were weighed, frozen, and homogenized in phosphate buffer. Homogenates were centrifuged at 10,000 × *g* for 10 min at 4°C and the supernatant was used in the Amplex red assay according to manufacturer instructions. Samples were measured with a 96-well microplate reader (Infinite 200 PRO; Tecan) using 530- and 590-nm excitation and emission filters. Fluorescence reads were then normalized to the leaf weight. Superoxide anion radical accumulation was detected by nitroblue tetrazolium chloride (NBT) (Sigma) as described previously (Basu et al. 2020), with some modifications. The 5th or 7th leaf was weighed and stained with freshly made NBT solution (1.0 mg of NBT per milliliter in 10 mM NaN₃ and 10 mM phosphate buffer, pH 7.5) for 1 h. NBT-stained samples were then ground and were used to quantify the generation of formazan, by spectrophotometric analysis at 700 nm, using a 96-well microplate reader (Infinite 200 PRO) in the case of data for *MSL10g* lines with lesions, whereas *MSL10* overexpression lines were all analyzed using BioTek PowerWave XS2 microplate spectrophotometer (BioTek). Absorbance reads were then normalized to the leaf weight.

Quantification of cell death.

Cell death was visualized in leaves of three- to five-week-old soil-grown *Arabidopsis* plants using trypan blue staining. Whole plant images of *MSL10* overexpression lines were obtained with an Olympus SZx7 Stereomicroscope with DP71 digital camera and fitted with DFPL 0.5×-4 objective, while trypan blue-stained lesions were imaged using a BX53 Olympus microscope with DP80 camera digital camera fitted with a UPlanFL N 10x/0.30 objective. The size of trypan blue-stained regions was quantified using ImageJ software as described (Fernández-Bautista et al. 2016; Basu et al. 2020). Cell death in tobacco leaves was determined by staining with FDA (500 µg/ml) and PI (1.25 µg/ml) for 15 min as described by Velej et al. (2014). The percentage of dead cells was calculated from randomly selected fields from at least three leaves per infiltration.

Genotyping.

DNA was extracted by grinding tissue in extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 250 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]) and precipitating with an equal volume of isopropanol. PCR-based genotyping of *msl10-3G*, *msl10-1*, and *msl9-1* alleles was performed as described by Haswell et al. (2008) and Basu et al. (2020).

Immunodetection.

Total proteins were extracted from leaf samples as described previously (Basu et al. 2020). Nonchlorotic leaves of 3- to 5-week-old transgenic *Arabidopsis* plants expressing MSL10-GFP or *N. benthamiana* leaves 1 week after infiltration with MSL10-GFP were collected. Tissue was homogenized in 2× sample buffer followed by centrifugation at 10,000 × *g* for 5 min. The supernatant was resolved by 10% SDS-polyacrylamide gel electrophoresis and was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) for 16 h at 100 mA. Transferred proteins were probed with anti-GFP (1:5,000 dilution) (Takara Bio) or anti-tubulin (1:20,000 dilution) (Sigma) as primary antibodies, with incubation for overnight or 2 h, respectively. In some cases, equal loading was demonstrated by staining the PVDF membrane with Coomassie brilliant blue R 250 (Sigma) post-immunodetection. Immunodetection was performed by incubating the immunoblot for 2 h with anti-mouse immunoglobulin G conjugated with horseradish peroxidase (1:10,000 dilution) (Millipore). Detection was performed using the SuperSignal West Dura detection kit (Thermo Fisher Scientific). Relative intensities were determined by comparing MSL10-GFP protein band intensity with the reference tubulin band intensity or Coomassie blue-stained RuBisCO at 56 kDa, using Image J. The quantification was performed on two replicate immunoblots, each using different plant samples. Statistical analysis was performed using Student's *t* test, and *P* values are provided in Supplementary Table S2. None of the experimental MSL10-GFP intensities were significantly different from controls.

BiFC assays.

Entry vectors containing various truncated versions of the *MSL10* coding region were recombined into the binary vector pDEST-VYCE(R)GW or pDEST-VYNE(R)GW (Gehl et al. 2009), which carry the C-terminal or N-terminal fragment of Venus YFP (yellow fluorescent protein), respectively, using LR Clonase. The PCR primers used are listed in Supplementary Table S1. MSL10 was tagged at the C terminus, whereas SGT1b, RAR1, and HSP90.1 were tagged at the N terminus. For subcloning, the open reading frames (ORFs) of *SGT1b*, *RAR1*, and *HSP90.1* were amplified from leaf complementary DNA using CloneAmp HiFi PCR Premix (Clontech) and were cloned into pENTR/D-TOPO (Life Technologies). Next, these ORFs were

inserted into the BiFC destination vector pDEST-VYNE(R)GW, using the Gateway LR Clonase II enzyme mix (Life Technologies). For MSL10, we used constructs previously generated by Basu et al. (2020). The plasmids were introduced into *Agrobacterium* sp. strain GV3101 and pairwise combinations were coinfiltrated into four- to six-week-old *N. benthamiana* leaves as described (Waadt and Kudla 2008). To suppress posttranscriptional gene silencing, each construct pair was coinfiltrated with *Agrobacterium* sp. strain AGL-1 harboring *p19* (Senthil-Kumar and Mysore 2014). Infiltrated abaxial leaf areas were examined for YFP signal using a confocal microscope (Olympus Fluoview FV 3000) at 3 to 5 days postinoculation, using excitation at 488 nm and collecting emission from 495 to 550. The experiments were performed at least three times using different batches of plants; for each independent experiment, three *N. benthamiana* plants were infiltrated.

Split-ubiquitin assay (mbSUS).

Determination of protein-protein interactions by the mbSUS assay was conducted as described previously (Basu et al. 2020; Lee et al. 2019; Obrdlík et al. 2004). The coding regions of *SGT1b*, *RAR1*, and *HSP90.1* cloned into the BiFC destination vectors described above were used as template for amplification, using universal primers as described by Obrdlík et al. 2004). For MSL10, the construct used was described by Basu et al. (2020). The primers used are listed in Supplementary Table S1. These PCR products were cotransformed with either *pMetYCGate* or *pXNGate21-3HA* yeast vectors into THY.AP4 or THY.AP5 yeast strains, respectively. All yeast vectors and strains were obtained from the Arabidopsis Biological Resource Center (ABRC). Strength of interaction was quantified by β -galactosidase activity assays as described (Zhou et al. 2015).

Pseudomonas infection assays.

Bacterial strains *P. syringae* DC3000, *P. syringae* COR⁻ (DB29; *cmA cfa6* [Brooks et al. 2004]) and the *hrpL* mutant (Zwiesler-Vollick et al. 2002) were donated by B. N. Kunkel (Department of Biology, Washington University, St Louis). *P. syringae* bacterial strains were cultured on King's B (KB) media plates supplemented with rifampicin at 50 mg ml⁻¹ for *Pto* DC3000 and kanamycin at 50 mg ml⁻¹ for the *P. syringae* pv. *tomato* COR⁻ mutant. For *P. syringae* DC3000 and *hrpL* mutant growth assays, syringe-infiltration was performed as described by Liu et al. (2015). Briefly, 10⁵ CFU per milliliter (optical density at 600 nm [OD₆₀₀] = 0.0002) in 10 mM MgCl₂ were injected into the underside of the leaves of 5-week-old plants, using a needleless 1-cc syringe. For the *P. syringae* COR⁻ mutant, spray-inoculation was performed at a bacterial titer of OD₆₀₀ = 0.2, which corresponds to the final inoculum concentration of 1 × 10⁸ CFU (Jacob et al. 2017). After the indicated days of infection, leaf discs were obtained from three separate plants belonging to each genotype per bacterial strain tested. Bacterial titer was determined by grinding leaf discs to homogeneity in 10 mM MgCl₂, using a bead mill, and plating a dilution series onto KB plates with appropriate selection. Colonies were counted, the number was used to calculate the mean CFU per square centimeter for each treatment, and the values were log-transformed. The log-transformed means from individual biological replicates, as single datapoints, were then combined from three to five independent biological trials and used to calculate the mean and standard error.

VIGS and *Agrobacterium*-mediated transient assays.

Plasmids pTRV1 and pTRV2 were used for VIGS assays, as previously described (Siddiqui et al. 2008). Constructs pTRV2:00, pTRV2:*NbSGT1*, pTRV2:*NbRAR1*, and pTRV2:*LeHSP90* were obtained from the ABRC. The empty vector pTRV2:00 was used as

a negative control. pTRV2:*NbPDS* (*PHYTOENE DESATURASE*) was used as a positive control for silencing. All these constructs were transformed into *A. tumefaciens* strain GV3101. For leaf infiltration, GV3101 harboring pTRV1 or transformed with one of the pTRV2 constructs were mixed at a 1:1 ratio to a final OD₆₀₀ of 0.5 and were then coinfiltrated into two-week-old *N. benthamiana* leaves. Silencing efficiency was evaluated 9 days after infiltration by performing RT-qPCR analysis. After confirming suppression of *SGT1*, *RAR1*, and *HSP90*, infiltration of MSL10-GFP, *MSL10^{4A}-GFP*, and *MSL10^{4D}-GFP* constructs was performed to a final OD₆₀₀ of 0.5, along with *p19* to a final OD₆₀₀ of 0.3, as described by Maksaev et al. (2018). Each silencing experiment was repeated three times, and each experiment included at least three different plants.

Stomatal aperture.

Plants were grown under short-day conditions for five to six weeks. Intact leaves (5th, 7th, or 9th) were used for aperture measurements as described (Eisele et al. 2016). Leaves were exposed to white light for 1 h while floating (abaxial side touching the solution) in stomatal incubation buffer containing 10 mM MES-KOH, 10 mM KCl, 50 μ M CaCl₂, pH 6.5. Subsequently, one-half of a leaf was immersed in stomatal incubation buffer with the bacterial suspension at 1 × 10⁸ CFU/ml, OD₆₀₀ = 0.2 and incubated for 4 h; whereas the other half of the leaf was incubated in water. Abaxial leaf surfaces were imaged, and stomatal apertures were measured with ImageJ, as described (Chitrakar and Melotto 2010). To monitor stomatal closing in response to ABA, leaf discs with pre-opened stomates were exposed to the same buffer supplemented with 10 μ M ABA for 2 h under the same conditions used by An et al. (2016). Independent trials of the experiments were done around the same time of the day for each of the independent trials and the treatments to avoid potential rhythmic effects of photoperiod on stomatal aperture.

Confocal microscopy.

The subcellular localization of MSL10-GFP fusion proteins in epidermal cells of *N. benthamiana* leaves and *Arabidopsis*, as well as FDA and PI dual-stained tobacco epidermal cells, were visualized using an inverted FV3000 (Olympus) confocal microscope using a 20 $\times$ objective. The percentage of dead cells was quantified as outlined by Velej et al. (2014) and Maksaev et al. (2018). FDA and PI dual-stained epidermal cells were imaged sequentially, using the 488-nm laser to excite FDA and the 561-nm laser to excite PI. Emission was detected between 505 and 525 nm and between 580 and 660 nm for FDA and PI, respectively. The setting used for capturing FDA signals was also used for detecting GFP signal.

Statistical analyses.

Statistical evaluations for all figures were conducted using R Studio software (RStudio) and GraphPad Prism version 7.0. Statistical differences were analyzed as indicated in the figure legends. Tukey's highly significant difference post hoc test was used to determine statistical significance for balanced data sets. Scheffe's post hoc test was used to determine statistical significance for unbalanced data sets.

Data availability.

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative database or GenBank under the following accession numbers: *MSL9* (At5g19520), *MSL10* (At5g12080), *SAG12* (At5g45890), *PERX34* (At3g49120), *DOX1* (At3g01420), *OSM34* (At4g11650), *UBQ5* (At3g62250), *EF1 α* (At5g60390), *PAD4* (At3g52430), *SID2* (At1g74710), *SGT1b* (At4g11260), *HSP90.1* (At5g52640), *RAR1* (At5g51700), *EDS1* (At3g48090), *NDRI* (At3g20600), and *PR1* (At2g14610).

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LITERATURE CITED

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