Mechanosensing and Sphingolipid-Docking Mediate Lipopeptide-Induced Immunity in *Arabidopsis*

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Abstract

Bacteria-derived lipopeptides are immunogenic triggers of host defenses in metazoans and plants. Root-associated rhizobacteria produce cyclic lipopeptides that activate systemically induced resistance (IR) against microbial infection in various plants. How these molecules are perceived by plant cells remains elusive. Here, we reveal that immunity activation in *Arabidopsis thaliana* by the lipopeptide elicitor surfactin is mediated by docking into specific sphingolipid-enriched domains and relies on host membrane deformation and subsequent activation of mechanosensitive ion channels. This mechanism leads to host defense potentiation and resistance to the necrotroph *B. cinerea* but is distinct from host pattern recognition receptor-mediated immune activation and reminiscent of damage-induced plant immunity.

Main Text

Lipopeptides (LPs) represent a prominent and structurally heterogeneous class of molecules among the broad spectrum of small specialized metabolites synthesized by bacteria. Besides serving key functions for the ecological fitness of the producer (motility, biofilm formation, colonization, nutrient acquisition, or antagonism towards competing neighbors), some LPs also act as triggers of immune responses that restrict pathogen infection of metazoans and plants[1,2]. The vast majority of LPs formed by plant-associated bacteria are comprised of a partly or fully cyclized oligopeptide linked to a single fatty acid chain. Some of these cyclic lipopeptides (CLP) formed by beneficial species belonging to the *Pseudomonas* and *Bacillus* genera are potent elicitors of immune responses in the host plant leading to a systemically induced resistance (IR) against infection by microbial pathogens[2,3].

67 This CLP-induced plant resistance is a key process for biocontrol of crop diseases [4], but, in
68 contrast to pattern-triggered immunity (PTI), its molecular basis remains poorly understood.
69 Like in animals, PTI in plants relies on the detection of specific molecular motifs (Microbe-
70 Associated Molecular Patterns (MAMPs) via cell-surface plasma membrane (PM)-localized
71 Pattern-Recognition Receptors (PRRs)[5]. Upon assembly of higher order receptor complexes
72 involving conserved co-receptors, PRRs activate receptor-like cytoplasmic kinases (RLCKs)
73 such as BIK1 and its closest homolog PBL1 described as key convergent signaling hubs. This
74 leads to phosphorylation of numerous substrate proteins and subsequent induction of a
75 well-characterized immune response[6]. Early hallmarks of PTI signaling in plants include
76 apoplastic burst of reactive oxygen species ($[ROS]_{apo}$), calcium influx, medium alkalinization
77 indicating H^+/K^+ exchange and membrane depolarization, MAPK phosphorylation cascade
78 and initiation of transcriptional reprogramming [7,8,9,10].

79 The CLP surfactin (Srf, **Fig 1A**) is well conserved in plant beneficial bacilli[11] and is
80 among the bacterial compounds best described as immunity elicitor in several plant
81 species[2]. In *Arabidopsis thaliana* ecotype Col-0 (hereafter, *Arabidopsis*), root treatment with
82 purified Srf (at 10 µM as minimal active concentration previously determined[12] and used as a
83 mix of naturally produced homologues slightly differing in the length of the fatty acid tail,
84 see **Suppl Fig 1**) triggers IR and significantly reduces leaf infection by the grey mold pathogen
85 *Botrytis cinerea* (**Fig 1B**). Therefore, we used Srf as a model to further investigate the
86 molecular mechanisms determining CLP perception and immunity stimulation in *Arabidopsis*
87 root cells.

88 We first performed quantitative and time-resolved measurements of early responses
89 commonly associated with MAMP perception in *Arabidopsis* and other plants. $[ROS]_{apo}$ burst
90 is almost invariably associated with PTI[7] but, by contrast to treatment with the MAMP

91 flagellin-derived peptide flg22 or with chitin, we did not observe a $[ROS]_{apo}$ burst in
92 *Arabidopsis* root cells treated with Srf based on a horseradish peroxidase-luminol assay
93 (**Suppl Fig 2**). Srf-mediated IR against *B. cinerea* is fully conserved in the *rbohD* mutant
94 lacking functional plasma membrane NADPH oxidase RBOHD responsible for MAMP-induced
95 $[ROS]_{apo}$ burst[13] (**Suppl Fig 3**). Hence, Srf-mediated activation of IR in the root does not
96 require RBOHD[14]. However, Srf triggered a fast and consistent increase in intracellular ROS
97 ($[ROS]_{intra}$) in root loaded with the fluorescent probe DCFH-DA (**Fig 1C**). This Srf-triggered
98 $[ROS]_{intra}$ burst is also observed in the *rbohD* mutant (**Suppl Fig 4**), suggesting it is not caused
99 by the uptake of apoplastic ROS via aquaporins (see **Suppl Fig 5** for response to flg22) but
100 may originate from different organelles as reported for abiotic stresses or other small
101 microbial compounds[15,16,7,17]. Calcium influx typically associated with PTI in plants[8] was
102 tested upon elicitation by Srf using an aequorin-based bioluminescence assay. It did not
103 reveal any significant Ca^{2+} increase ($[Ca^{2+}]_{cyt}$) in root of the Col-0^{AEQ} reporter line in contrast
104 to the increase observed upon flg22 treatment (**Fig 1D**) or in response to chitin (**Suppl Fig 6**).
105 On the other hand, medium alkalinization occurs within minutes after Srf treatment (**Fig 1E**),
106 which indicates H^+/K^+ exchange possibly leading to membrane depolarization[9]. However, no
107 significant increase in conductivity was measured in the medium following Srf treatment
108 (**Suppl Fig 7**) indicating that the lipopeptide does not affect plasma membrane (PM) integrity
109 and does not cause massive electrolyte leakage. Cell viability tests confirmed that Srf is not
110 toxic for *Arabidopsis* root cells at concentrations up to 50 µM (**Suppl Fig 8**).

111 Next, we explored early changes in the root transcriptome profile induced by Srf via
112 time course RNAseq analysis (30 min, 1h, 3h and 6h post treatment) using the same setup
113 previously reported for flg22 and the fungal MAMP chitin[18]. Data revealed a relatively low
114 transcriptional response to Srf elicitation over all sampling times with a total of 564

115 differentially expressed genes (DEGs, Log_2 Fold Change > 2, p<0.05; **Fig 1F**) compared to
116 approximately 5000 DEGs and 2000 DEGs reported upon flg22 and chitin treatment
117 respectively[18]. While MAMPs mainly up-regulate early responsive genes (30 min – 1 h)[18,19],
118 an almost equal number of up- and down-regulated DEGs were observed upon Srf treatment
119 at all time points (**Fig 1F**), with about half of the transcriptional changes specific to Srf
120 elicitation (47,9% and 58% compared with flg22 and chitin respectively)[18]. Strikingly, many of
121 the Srf down-regulated genes are upregulated by flg22 and chitin[18] (**Fig 1F, Suppl Table 1**).
122 Differential expression was confirmed by quantitative RT-PCR performed on some selected
123 genes in plantlets elicited with the lipopeptide and with chitin (**Suppl Fig 9**). More
124 specifically, the expression of genes typically associated with early immune signaling
125 (receptor-like kinases, $[ROS]_{apo}$ burst, calcium signaling or MAPK phosphorylation cascade[10])
126 or defense mechanisms (pathogenesis-related (PR) proteins, callose deposition, lignification)
127 is not modulated or down-regulated by Srf by contrast with MAMP treatment (**Fig 1G, Suppl**
128 **Table 1**).. However, *CYP71A12*, encoding a key enzyme of the camalexin biosynthesis
129 pathway[20], is among the late-responsive genes (6h) strongly stimulated by Srf. In
130 accordance, we measured significantly higher amounts of this phytoalexin, which is toxic to
131 *B. cinerea*[21,22], in infected leaves of Srf-treated plants compared with mock treatment (**Fig**
132 **1H**). The key role of camalexin in disease control was confirmed by the loss of Srf-triggered
133 resistance in the *pad3* mutant[23] unable to form camalexin[22] (**Fig 1I**). Thus, by contrast to PTI
134 which is associated with substantial transcriptional reprogramming[19], immunity stimulation
135 by Srf does not lead to major changes in the expression of genes involved in signaling and
136 defense.

137 Since the molecular basis of Srf-induced immune activation is signal-specific, we
138 hypothesized that plant cells perceive lipopeptides by a mechanism that differs from pattern

139 sensing. Srf possesses both a peptidic moiety and a fatty acid tail, but its IR-eliciting potential
140 is fully conserved in *Arabidopsis* mutants lacking functional PRRs that recognize either
141 bacterial proteinaceous immunogenic patterns or acyl chain epitopes such as medium chain
142 3-hydroxy fatty acids and HAAs[24,25] (**Suppl Fig 10**). Srf elicitation is not significantly affected
143 either in mutants lacking co-receptors required for proper functioning of a wider range of
144 PRRs detecting immunogenic peptides such as Pep1[26], nlp20[27] and IF1[28] nor in the *bik1 pbl1*
145 double mutant lacking RLCKs that act downstream of the PRR-co-receptor complexes (**Suppl**
146 **Fig 10**). Although we only tested a small subset of the multitude of PRRs potentially
147 expressed in *Arabidopsis*[6] and although early cellular signaling may be BIK1/PBL1-
148 independent[29], our data strongly suggest that *Arabidopsis* does not sense Srf via PRR-type
149 cell surface sentinels. This is in accordance with previous data from tobacco, which showed
150 that Srf is still active on protease-treated cells and that there is no refractory state upon
151 repeated Srf treatment unlike typically observed for PTI[30].

152 Due to their amphipathicity, CLPs readily interact with biological membranes, causing
153 pore formation and membrane disruption responsible for their antimicrobial activities[31].
154 Such an adverse effect is not expected on plant membranes, but we hypothesized that Srf
155 perception by root cells might primarily rely on its interaction with the lipid phase of the PM.
156 Complex sphingolipids glucosylceramides (GluCer) and glycosyl inositol phosphorylceramides
157 (GIPC) constitute more than 30% of *Arabidopsis* PM lipids and are key components required
158 for membrane integrity and functionality, notably by forming ordered nano-domains with
159 sterols[32,33,34]. *In silico* docking simulation first revealed a more favorable interaction of Srf
160 with GluCer or GIPCs than with the other typical plant PM lipids PLPC (1-Palmitoyl-2-
161 linoleoyl-sn-glycero-3-phosphocholine as phospholipid) and ß-sitosterol (as main sterol) (**Fig**
162 **2A**). To test this experimentally, we generated biomimetic liposomes using commercially

163 available GluCer, PLPC and ß-sitosterol. Isothermal titration calorimetry performed on
164 liposomes with increasing composition complexity in such lipids showed the highest binding
165 affinity of Srf to model membranes containing GluCer (**Fig 2B**). In support of a preferential
166 interaction with sphingolipids, molecular dynamic (MD) simulation on the same ternary lipid
167 system showed the specific insertion of Srf in the vicinity of GluCer molecules or in GluCer-
168 enriched areas in the membrane (**Fig 2C**). In light of these results, we tested Srf elicitor
169 activity on the *Arabidopsis* ceramide synthase mutant *loh1* (LONGEVITY ASSURANCE 1
170 HOMOLOG1) which is depleted in these complex sphingolipids[35,36]. We observed strongly
171 reduced $[ROS]_{intra}$ responses (**Fig 2D**) as well as loss of IR to *B. cinerea* infection in *loh1*
172 compared to wild-type plants (**Fig 2E**). Such lipid-dependent $[ROS]_{intra}$ elicitation was also
173 observed for other IR-eliciting CLPs such as orfamide and WLIP[2] isolated from beneficial
174 pseudomonads that resemble Srf in size and amphiphilic character (**Suppl Fig 11**). The CLP
175 immunogenic activity thus relies on an intricate interaction with PM sphingolipids as
176 reported for other microbial compounds[36,37,38].

177 By inserting into lipid bilayers, Srf may transiently affect the local structure of
178 membranes. Indeed, neutron reflectivity (NR) experiments (see **Suppl Fig 12** for deuterated
179 Srf synthesis and characterization) demonstrate that Srf exclusively inserts into the outer
180 leaflet of PLPC-ß-sitosterol-GluCer model membranes (**Fig 3A**). This is supported by MD
181 simulation showing that the Srf peptide backbone preferentially positions at the level of the
182 polar lipid heads of the membrane (**Suppl Fig 13**). Srf insertion does not affect the lipid
183 chain-chain interaction as shown by WAXS and FTIR (**Suppl Fig 13**). In addition, NR data
184 indicated that Srf insertion results in a decrease in membrane thickness (from 40 to 36Å),
185 which is more pronounced in ternary membranes than in membranes lacking GluCer (from
186 43 to 41Å) (**Suppl Table 4**). Analysis of the nanoscale morphology of supported PLPC-ß-

187 sitosterol-GluCer bilayers by Atomic Force Microscopy confirmed this membrane thinning
188 caused by Srf insertion (**Suppl Fig 14**). An additional impact of the lipopeptide on PM
189 physical properties was derived from coarse-grained MD simulation which revealed a strong
190 curvature-inducing effect mediated by Srf docking on ternary membranes (**Fig 3B**).

191 In light of these biophysical data, a clear impact of Srf on PM structure can be
192 predicted but in integral root cells, the PM is physically connected to the thick and
193 mechanically strong cell wall polymer matrix, which provides structural support and might
194 stabilize the membrane into a flat conformation under low tension[39]. We thus next tested
195 early Srf-induced immune responses in cell wall-free protoplasts. Use of PPs renders the PM
196 more susceptible to deformation, which was used to study responses to cell swelling or
197 shrinkage/expansion during osmotic stresses[40,41]. As in root cells, Srf triggered a consistent
198 $[ROS]_{intra}$ burst in freshly isolated protoplasts (**Suppl Fig 15**). However, in contrast to roots, a
199 significant calcium influx, was observed in Srf-treated protoplasts by using the Col-0^{AEQ}
200 reporter line and also by loading Col-0 with the Fluo4-AM probe (**Fig3C and Suppl Fig 16**).
201 This Srf-induced Ca^{2+} influx is comparable in amplitude to the one induced by MAMPs (**Suppl**
202 **Fig 17**). It involves some PM channels since it is abolished in protoplasts pre-treated with the
203 general channel blocker $LaCl_3$ (**Suppl Fig 18**) and considering that the lipopeptide does not
204 cause any detrimental effect on protoplast viability at the concentration used (**Suppl Fig 19**).
205 Additional assays on protoplasts revealed that activation of early responses by Srf requires
206 threshold concentrations of 5-10 µM both for calcium influx (**Fig 3D**) and $[ROS]_{intra}$ burst
207 (**Suppl Fig 20**), which is much higher than MAMPs detected at nanomolar concentrations.
208 This further indicates that Srf perception is not mediated by a high-affinity receptor-based
209 detection system and is in accordance with the mechanism predicted from biophysics in
210 which threshold amounts of Srf molecules must dock into sphingolipid domains in order to

211 modulate PM structure. We tested the impact of the lipopeptide on protoplast membrane
212 fluidity via measurements of laurdan generalized polarization (laurdan GP) related to the
213 lipid bilayer order. Our results show that Srf treatment led to a significant increase in ΔGP
214 values indicating a clear membrane rigidification effect as also observed upon interaction of
215 the lipopeptide with PM mimicking liposomes (**Fig 3E**).

216 Altogether, these data obtained with protoplasts support the relevance of PM
217 deformation in the response to Srf. We therefore hypothesized that insertion of the CLP
218 could induce physical constrains resulting in increased lateral tension sufficient for activating
219 mechano-sensitive (MS) ion channels, in a process similar to the one observed for some
220 anionic amphipathic chemicals[42,43]. This was supported by the reduced calcium influx
221 observed upon pre-treatment of Col-0^{AEQ} protoplasts with the specific MS channel blocker
222 GsMTX-4 (**Suppl Fig 21**). Among stretch sensitive mechanosensors identified so far in plant
223 cells, MSL9, MSL10 and MCA1/2 localize in the PM [44,45,46] but do not require RLCK-mediated
224 phosphorylation of the cytoplasmic domains for gating unlike other MS ion channels such as
225 OSCA1.3 which needs BIK1 phosphorylation to be activated [47]. Using Fluo-4, we thus tested
226 protoplasts prepared from the quintuple *msl4/5/6/9/10*[48] and the double *mca1/2*[49] mutants
227 for their response to Srf and observed a significantly decreased calcium influx, to the same
228 extent as chemical inactivation with GsMTX-4 in Col-0 (**Fig 3F**).

229 We next evaluated the effect of inactivation or knock-out of *msl* and *mca* channels on
230 intracellular ROS burst as early response of root tissues elicited by Srf. Pre-treatment with
231 GsMTX-4, $LaCl_3$ or with the Ca^{2+} chelator EGTA eliminated the ROS burst triggered by Srf in
232 root tissues (**Fig 3G**), supporting the importance of MS channels in the response and
233 indicating that ion fluxes acts upstream of or are interdependent of $[ROS]_{intra}$[8]. An almost
234 complete loss of $[ROS]_{intra}$ burst was also observed upon Srf treatment in the *msl4/5/6/9/10*

235 and *mca1/2* mutants as compared to Col-0 (**Fig 3H**). In addition, *mca1/2* and *msl4/5/6/9/10*
236 plants were strongly impaired in mounting systemic resistance against *B. cinerea* upon Srf
237 treatment (**Fig 3I**), further indicating that functional MS channels are necessary for full
238 response of *Arabidopsis* to Srf elicitation on roots. Data on protoplasts show that Srf may
239 trigger some calcium transients as early immune-related event but a detectable Ca^{2+} influx is
240 not required for defense activation in plantlets, which correlates with the fact that no
241 downstream components of calcium signaling are up-regulated upon perception of the
242 lipopeptide.

243 Collectively, although contributions of other channels cannot be ruled out[47], our data
244 provides evidence for a key role of PM-located mechanosensors in lipopeptide-induced plant
245 defenses. The relative contribution of each channel remains to be determined as they
246 display specific properties in terms of sensitivity to membrane tension and ion selectivity.
247 MCA1/2 are described as genuine transporters of Ca^{2+} [46,50] while MSL10 is regarded as a non-
248 selective ion transporter that is indirectly involved in calcium signaling upon wounding[51] and
249 response to hypo-osmotic shock in cell swelling[41]. Both channels may thus act in a
250 coordinated fashion to tailor ion fluxes leading to cellular responses and PM depolarization.
251 As previously reported for other plant species[2], treatment with Srf prepares *Arabidopsis* to
252 mount defense responses culminating in the systemically expressed IR phenotype. We
253 provide new insights into the molecular basis of the well-known long-standing process of
254 CLP-triggered plant immunity activation by unveiling a new lipid-mediated mechanism for
255 the detection of these molecules at the cell surface. We infer from our data that CLP
256 insertion into sphingolipid-enriched PM domains causes deformation and increases lateral
257 tension in the membrane leading to rearrangement of the MS protein complexes and gating
258 of the channels. This allows ion influx and initiates chemical signaling that can be integrated

259 by root cells to activate early immune responses in a process that remains to be deciphered.
260 Such a lipid-dependent perception at the cell surface may apply also to other bacterial
261 amphiphilic IR elicitors such as acyl-homoserine lactones and rhamnolipids which also
262 readily interact with membrane lipids and may thus be perceived via similar
263 mechanisms[2,52,53,54,55]. The nature of PM lipids widely varies across plant species[34]. This could
264 explain, at least in the case of Srf, why this molecule triggers immunity in dicots but is not
265 very active on monocots[2]. We assume that the effect of a CLP on a particular target
266 membrane is also fine-tuned by precise structural traits in the molecule. It may explain why
267 some CLPs produced by *Pseudomonas* leaf pathogens act as virulence factors in a wide range
268 of plants by causing necrosis via pore formation in cellular membranes[56]. However, further
269 investigation is required to capture the physico-chemical rules governing lipid selectivity and
270 CLP insertion dynamics.

271 As the two components of the plant immune system, PTI works in concert with
272 effector-triggered immunity (ETI) for mounting robust defense responses to biotrophic
273 invaders but ETI is not efficient against necrotrophic pathogens[5,57]. Here, we describe a
274 novel molecular mechanism of defense activation in plants, which provides resistance to the
275 necrotroph *B. cinerea* via a unique process not related to the receptor-based surveillance
276 system involved in the recognition of MAMPs by plant cells or in the perception of the
277 Pam_3CSK_4 analog of triacylated lipopeptides produced by *Staphylococcus aureus* and acting
278 as agonists of Toll Like-type PRRs in metazoans[58]. It therefore provides new insights in plant-
279 microbe interactions mediated by small chemicals from beneficial bacteria. Collectively, our
280 data show that Srf perception leads to specific immune activation signature regarding the
281 type, timing, and amplitude of early defense-related events and the weak transcriptional
282 reprogramming as compared to PTI. This may explain why elicitation by Srf is cost-effective

283 for the host plant as it does not result in growth-defense trade-off[59,60] nor does it cause a
284 strong response associated with the alertness state or a hypersensitive reaction leading to
285 cell death. Using CLPs as elicitors would enable bacteria to bypass a strong immune response
286 and avoid their rejection as undesirable associate. Further investigations are needed for a
287 comprehensive understanding of the whole process from perception to systemic signaling
288 but the mechanistic basis of CLP-induced plant resistance reported here should contribute to
289 rationally implement the use of these compounds or their producers as bio-sourced
290 alternatives to chemicals in sustainable agriculture.
291

292 **Fig. 1. Surfactin triggers systemic resistance in *Arabidopsis* associated with atypical**
293 **immune responses. (A)** Structural model of the heptapeptide Srf (C14 acyl chain homologue)
294 in water (Gromacs v.4.5.4). Red: oxygen, white: hydrogen, dark blue: nitrogen, light blue:
295 carbon. The polar amino acids are circled in yellow, and other amino acids and the acyl chain
296 constitute the non-polar part of the molecule. **(B)** Disease incidence caused by *Botrytis*
297 *cinerea* in *Arabidopsis* Col-0 plants pre-treated with Srf (10 μM) or not (mock treatment, 0.1
298 % ethanol) (n=28 replicates from three independent experiments). The box plots encompass
299 the 1st and 3rd quartiles, the horizontal line indicates the median, and bars extend from the
300 lower to the higher values. Disease reduction (D.R.) is calculated from the mean values of
301 both treatments. Significant difference ***P<0.001, two-tailed t-test. **(C)** Burst in
302 intracellular ROS species in *Arabidopsis* Col-0 roots upon treatment with Srf (10 μM). Left,
303 time course of $[ROS]_{intra}$ accumulation (Relative Fluorescence Units, RFU) with data at each
304 time point representing mean ± SD, n=3 (independent root samples). Right, fold increase in
305 fluorescence values ± SD, at 30 min after the addition of Srf compared to mock-treated
306 roots. Data are pooled from three independent experiments (total n=9) and asterisks
307 indicate significant difference (***P<0.001, two-tailed t-test). **(D)** $[Ca^{2+}]_{cyt}$ kinetics in Srf-
308 treated (10 μM) or flg22-treated root tissues (1 μM) (n=6) compared to mock treatment in
309 the *Arabidopsis* Col-0^{AEQ} reporter line. Results are represented as luminescence counts per
310 second relative to total luminescence counts remaining (L/Lmax; mean ± SD). Experiments
311 were repeated three times with similar results. **(E)** pH variation in Col-0 root medium
312 following mock treatment or addition of 10 μM Srf. Values on the graph are normalized to
313 pH of the first time point ± SD and are from one representative experiment (n=4) out of 2
314 independent experiments showing similar results. **(F)** Number of DEGs (Log_2 Fold Change > 2,
315 P<0.05) in *Arabidopsis* root cells determined via RNAseq for each time point in response to

316 Srf treatment (10 μM). Our data were compared with those reported for DEGs in response
317 to flg22 (1 μM) and chitin (Chi, 1 mg/ml)[18] and bars are subdivided by the number of genes
318 specifically responding to Srf and by the number of genes differentially (oppositely)
319 regulated by Srf and the two MAMPs. **(G)** Heatmap of the expression of genes putatively
320 associated with plant immune responses (listed in Supp table 1) that were modulated upon
321 Srf treatment (S, left) (10 μM) and compared with their expression in response to flg22 (1
322 μM) and chitin (1 mg/ml) (F and C respectively, right) based on published data[18]. Colour
323 scale represents Log_2 FC (> 2, P<0.05). **(H)** Camalexin response associated with IR triggered
324 by Srf. Camalexin accumulation 96 hours post *B. cinerea* inoculation (hpi) in *Arabidopsis* Col-
325 0 leaves of mock- or 10 μM Srf-treated plants at the root level. Graph shows values obtained
326 in one experiment with each value representing a sample of five plants pooled together.
327 Asterisks indicate significant difference with ns, not significant; **P*<0.05; ****P*<0.001; two-
328 tailed *t*-test. **(I)** Disease incidence of *B. cinerea* in *pad3* mutant pretreated with 10 μM Srf or
329 mock-treated at the root level (n=30, values obtained from three independent experiments,
330 presented as differently shaded grey values). Data are represented as in fig 1B. Asterisks
331 indicate significant difference with ns, not significant; **P*<0.05; ****P*<0.001; two-way ANOVA
332 and Sidak's multiple-comparison post-test.
333
334 **Fig. 2. Affinity for sphingolipids determines CLP-triggered immunity. (A)** *In silico* docking
335 simulation of the interaction between Srf and plant PM lipids with their associated energy of
336 interaction (E_{int}). A lower E_{int} value indicates a more favorable interaction. Hydrogen, oxygen
337 and phosphate atoms are respectively represented in grey, red and blue. Carbon atoms of
338 Srf are in yellow and carbon atoms of GluCer, Sito and PLPC are in pink. **(B)** Binding
339 coefficient (K) of Srf to liposomes with different lipid compositions. Graph presents values

340 from two independent experiments, mean ± SD. **(C)** Molecular dynamics simulation of Srf
341 insertion in GluCer-enriched domains of a PLPC-Sito-GluCer bilayer. Left: Top views of
342 bilayers before and after Srf insertion (right). **(D)** $[ROS]_{intra}$ accumulation in roots of Col-0 and
343 *loh1* mutant. Data represents fold increase in fluorescence values ± SD (n=6 from two
344 independent experiments) at 30 min after Srf addition (10 μM) or not . Significant difference
345 ***P<0.001, two-tailed *t*-test. **(E)** Disease incidence of *B. cinerea* in *Arabidopsis* Col-0 and
346 *loh1* mutant plants, pre-treated with Srf (10 μM) compared with mock treatment (n=30 from
347 two independent experiments). Data are represented as in fig 1B. ns = not significant,
348 ***P<0.001, two-way ANOVA and Sidak's multiple-comparison post-test.
349

350 **Fig. 3. Srf causes membrane deformation and activates mechanosensitive channel-**
351 **dependent immune responses. (A)** Membrane thickness determined via neutron scattering
352 length density (SLD) profiles of supported PLPC-Sito-GluCer membrane before (black) and
353 after (green) Srf addition to the final 95:5 membrane: Srf molar proportion (0.24 μM)
354 (below). Illustration (above) presents the correspondence between regions in the SLD profile
355 and specific zones in the membrane. **(B)** Molecular dynamics simulation of Srf-induced
356 membrane curvature. **(C)** Left, $[Ca^{2+}]_{cyt}$ kinetics in Srf-treated (10 μM) root cell protoplasts
357 (n=6) compared to mock treatment in the *Arabidopsis* Col-0^{AEQ} reporter line. Results are
358 represented as luminescence counts per second relative to total luminescence counts
359 remaining (L/Lmax; mean ± SD). Experiments were repeated three times with similar results
360 (see Suppl Fig 16 for additional experiments). Right, increase in $[Ca^{2+}]_{cyt}$ detected upon
361 loading root protoplasts of Col-0 with Fluo-4 in mock- or Srf-treated (10 μM). Experiments
362 were repeated three times with similar results. **(D)** Dose-dependent $[Ca^{2+}]_{cyt}$ increase
363 induced by Srf in root protoplasts of *Arabidopsis* Col-0^{AEQ}. Values are the average of L/Lmax

364 values from 1.5 to 4 min after treatment corresponding to the top of the peak. Mean ± SD of
365 at least 10 technical replicates from at least five independent experiments. Asterisks indicate
366 statistically significant differences to the mock treatment (ns= no significant difference;
367 *P<0.05; ***P < 0.001; (a) two-tailed t-test; (b) Welch and Brown-Forsythe ANOVA). **(E)**
368 Change of laurdan generalized polarization (ΔGP) in Srf-treated (10 μM) Col-0 root
369 protoplasts and in liposomes reflecting a change of membrane rigidity. ΔGP is defined as the
370 subtraction of GP measured at 10 min following treatment and GP measured before
371 treatment. Mean ± SD of 12 (for protoplasts) and 15 (for liposomes) replicates from 8 (for
372 protoplasts) and 5 (for liposomes) independent experiments. ***P<0.001, two-way ANOVA
373 and Sidak's multiple comparison test. **(F)** $[Ca^{2+}]_{cyt}$ response measured with Fluo-4 upon Srf
374 elicitation (10 μM) in *Arabidopsis* Col-0 root protoplasts with and without pre-treatment
375 with the mechanosensitive channel blocker GsMTX-4 (10 min incubation, 7.5 μM)(n=10) and
376 in root protoplasts of the *mca1/2*, and *msl4/5/6/9/10* mutants (n=14). Mean ± SD from four
377 independent experiments. Letters represent statistically different groups at α = 0.05 (two-
378 way ANOVA and Tukey's multiple-comparison post-test). **(G)** $[ROS]_{intra}$ accumulation upon
379 addition of 10 μM Srf to *Arabidopsis* Col-0 roots upon pre-treatment or not (Col-0, n=16)
380 with the mechanosensitive channel blocker GsMTX-4 (10 min incubation, 7.5 μM) (n=12),
381 with the non-selective Ca^{2+} channel blocker $LaCl_3$ (10 mM) (n=7) and the Ca^{2+} chelator EGTA
382 (1 mM) (n=7). Data represent fold increase in fluorescence values 30 min after Srf addition
383 compared to mock-treated roots. Mean ± SD calculated from data from two independent
384 experiments. Letters represent statistically different groups at α = 0.05 (two-way ANOVA
385 and Tukey's multiple-comparison post-test). **(H)** $[ROS]_{intra}$ accumulation in *Arabidopsis* Col-0
386 (n=6), *mca1/2* (n=7), and *msl4/5/6/9/10* (n=8) roots following Srf treatment (10 μM). Data
387 represent fold increase in fluorescence values 30 min after Srf addition compared to mock-

388 treated roots. Mean ± SD from two independent experiments. ***P*<0.01, two-tailed *t*-test. **(I)**
389 Disease incidence of *B. cinerea* in *Arabidopsis* Col-0, *mca1/2*, and *msl4/5/6/9/10* mutant
390 plants, mock- or Srf pre-treated (10 μM)(each n=30 from two independent experiments
391 represented as differently shaded grey values). Data are represented as in fig 1B.

392

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407

408 **Author contributions**

409 J.P., G.G., M.D. and M.O. conceived and designed experiments; J.P., G.G., H.I., A.A., V.R.,
410 W.P.L-L, E.D., M.N.N., M.M-G., S.E., A.K., P.B. and Y.F.D. performed experiments and analyzed
411 data; J-M.C., M.G. and L.L. performed modeling; S.D., B.D.C, S.R., T.N., S.R., M.H. and C.Z.
412 substantially revised the manuscript and were involved in the discussion of the work; M.D.
413 and M.O. supervised the study and provided funding.

414

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416

417 **References**

418

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564

Figure 1

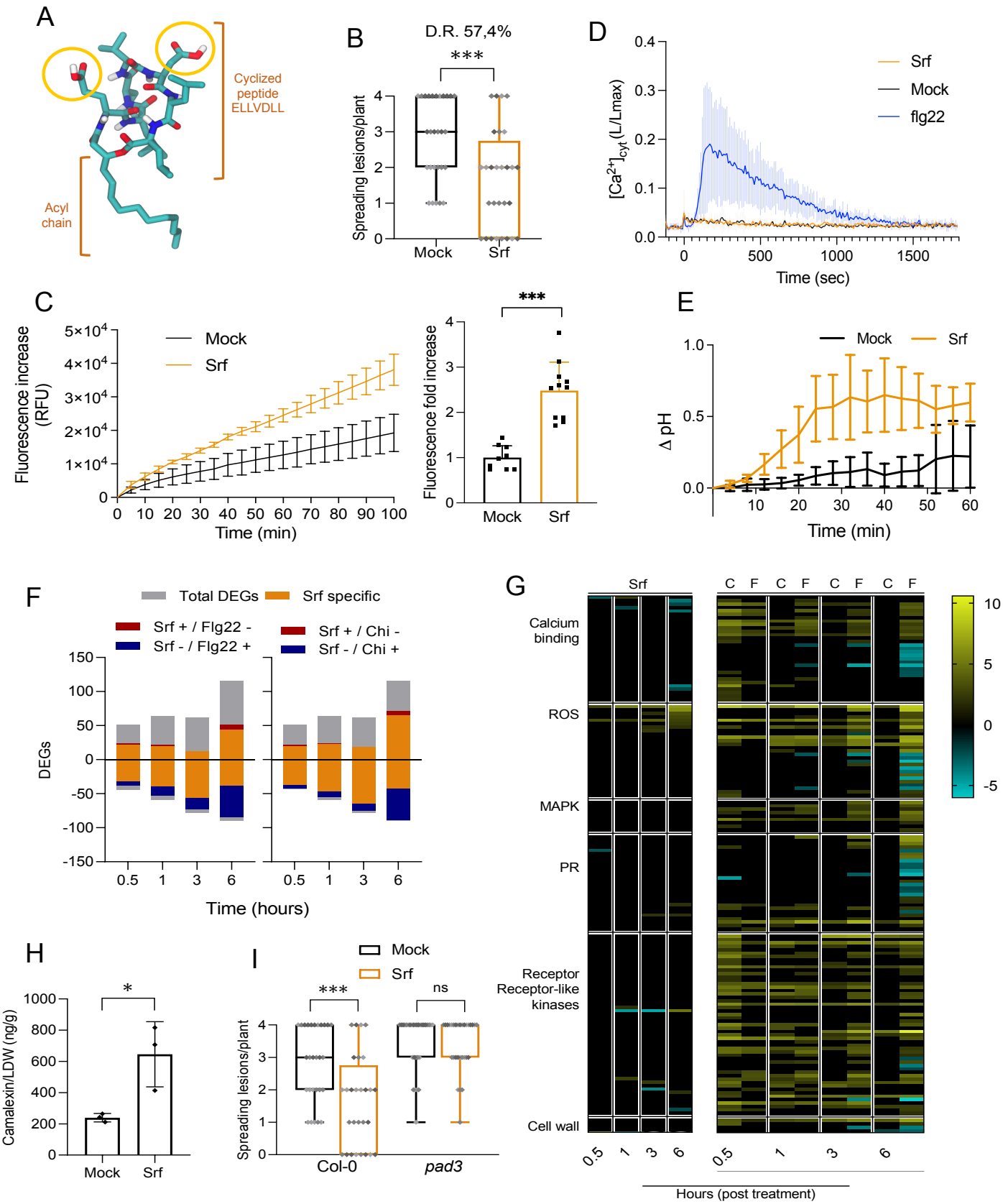
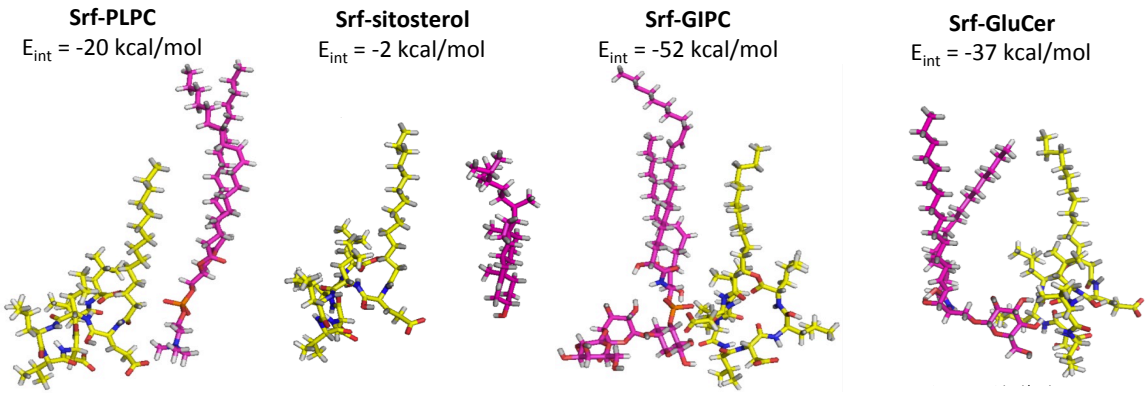
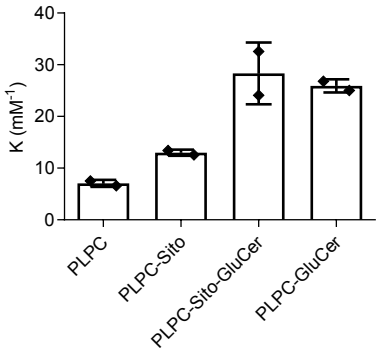

A
Cyclized peptide ELLVDLL
Acyl chain
B
D.R. 57,4%
***
Spreading lesions/plant
Mock
Srf
D
Srf
Mock
flg22
[Ca2+]cyt (L/Lmax)
Time (sec)
C
Mock
Srf
Fluorescence increase (RFU)
Time (min)
***
Fluorescence fold increase
Mock
Srf
E
Mock
Srf
Δ pH
Time (min)
F
Total DEGs
Srf specific
Srf + / Flg22 -
Srf - / Flg22 +
Srf + / Chi -
Srf - / Chi +
DEGs
Time (hours)
G
Srf
C F C F C F C F
Calcium binding
ROS
MAPK
PR
Receptor Receptor-like kinases
Cell wall
Hours (post treatment)
H
*
Camalexin/LDW (ng/g)
Mock
Srf
I
Mock
Srf
***
ns
Spreading lesions/plant
Col-0
pad3

Figure 2

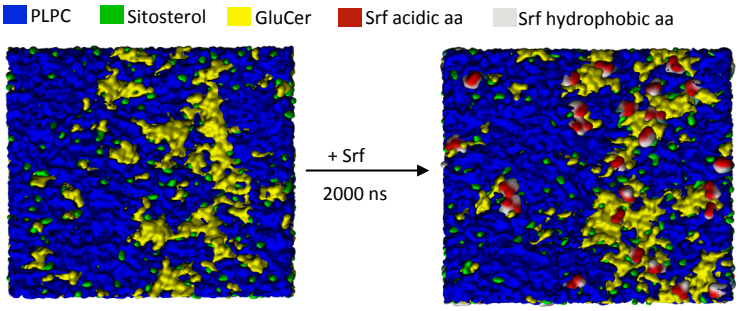
A



B



C



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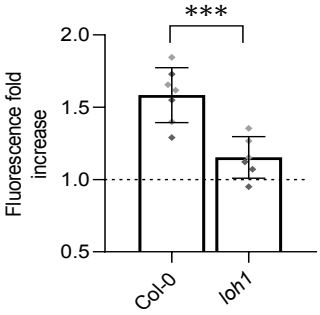

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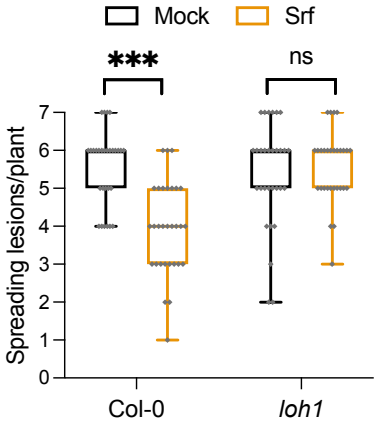
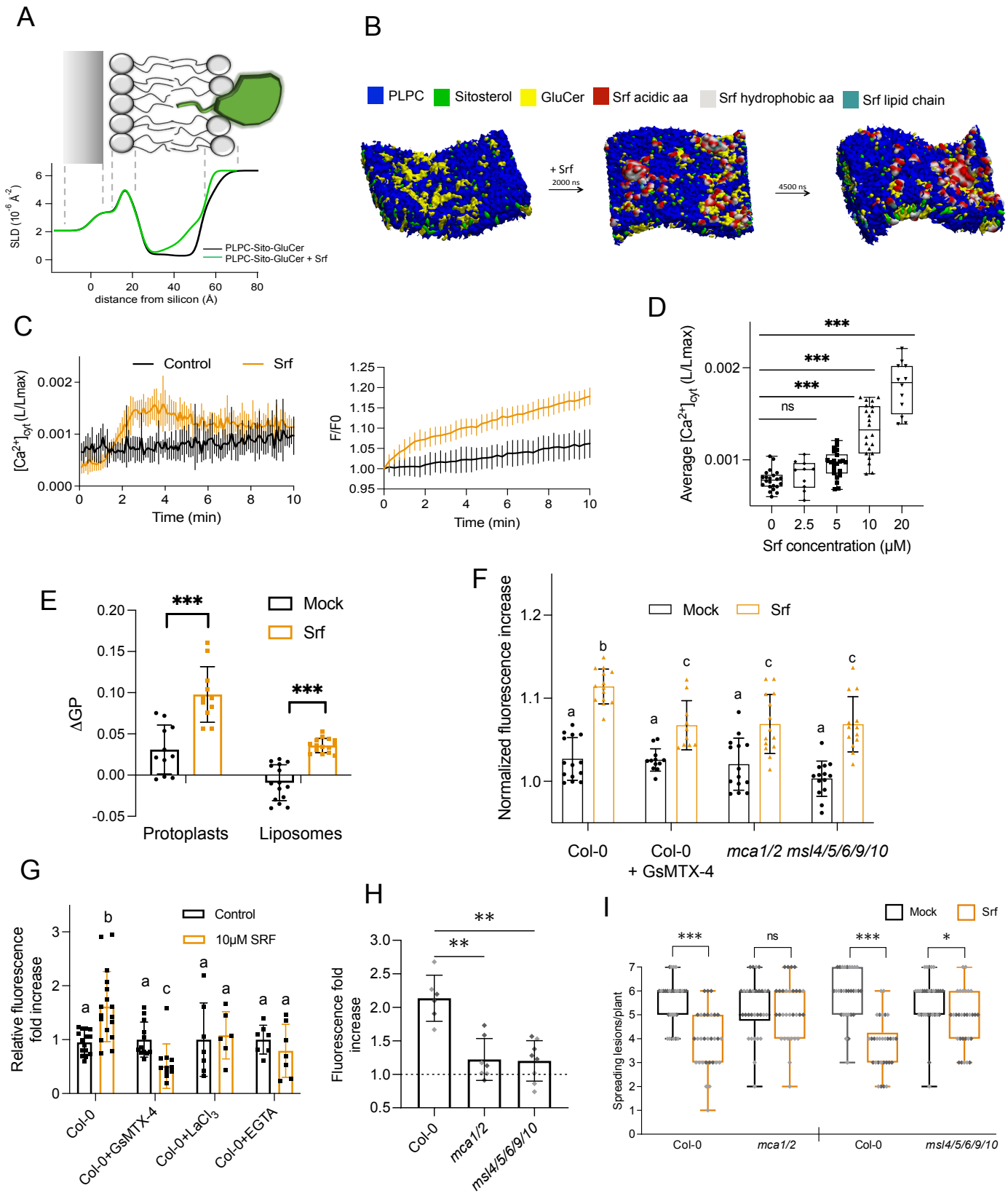


Figure 3


A
SLD (10^{-6} Å^{-2})
PLPC-Sito-GluCer
PLPC-Sito-GluCer + Srf
distance from silicon (Å)
B
PLPC
Sitosterol
GluCer
Srf acidic aa
Srf hydrophobic aa
Srf lipid chain
+ Srf
2000 ns
4500 ns
C
Control
Srf
$[Ca^{2+}]_{cyt}$ (L/Lmax)
Time (min)
F/F0
Time (min)
D
Average $[Ca^{2+}]_{cyt}$ (L/Lmax)
Srf concentration (µM)
E
Mock
Srf
ΔGP
Protoplasts
Liposomes
F
Mock
Srf
Normalized fluorescence increase
Col-0
Col-0 + GsMTX-4
mca1/2 msl4/5/6/9/10
G
Control
10µM SRF
Relative fluorescence fold increase
Col-0
Col-0+GsMTX-4
Col-0+LaCl3
Col-0+EGTA
H
Fluorescence fold increase
Col-0
mca1/2
msl4/5/6/9/10
I
Mock
Srf
Spreading lesions/plant
Col-0
mca1/2
Col-0
msl4/5/6/9/10