1 **The bacterial quorum sensing signal DSF hijacks *Arabidopsis thaliana* sterol biosynthesis to**

2 **suppress plant innate immunity**

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32 **Classification**

33   BIOLOGICAL SCIENCES/ Plant Biology

34

35

Abstract

36
37   Quorum sensing (QS) is a recognized phenomenon that is crucial for regulating population-

38 related behaviors in bacteria. However, the direct specific effect of QS molecules on host biology is

39 largely under-studied. In this work, we show that the QS molecule DSF (*cis*-11-methyl-dodecenoic acid)

40 produced by *Xanthomonas campestris* pv. *campestris* can suppress pathogen-associated molecular

41 pattern (PAMP)-triggered immunity (PTI) in *Arabidopsis thaliana*, mediated by flagellin-induced activation

42 of flagellin receptor FLS2. The DSF-mediated attenuation of innate immunity results from the alteration of

43 oligomerization states and endocytic internalization of plasma membrane FLS2. DSF altered the lipid

44 profile of *Arabidopsis*, with a particular increase of the phytosterol species, which impairs the general

45 endocytosis pathway mediated by clathrin and FLS2 nano-clustering on the plasma membrane. The DSF

46 effect on receptor dynamics and host immune responses could be entirely reversed by sterol removal.

47 Together, our results highlighted the importance of sterol homeostasis to plasma membrane organization

48 and demonstrate a novel mechanism by which pathogenic bacteria use their communicating molecule to

49 manipulate PAMP-triggered host immunity.

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51 Keywords: quorum sensing, plasma membrane, endocytosis, plant-pathogen interaction

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53 **SIGNIFICANCE STATEMENT**

54   Bacteria rely on small signalling molecules called quorum sensing (QS) signals to communicate

55 and coordinate their behaviors. QS is known to regulate gene expression, production of virulence factors,

56 and biofilm formation for pathogenic bacteria to effectively colonize their hosts and cause diseases. In this

57 work, we found a class of QS molecule called diffusible-signal factor (DSF), produced by devastating

58 phytopathogenic bacteria such as *Xanthomonas* spp. and *Xylella fastidiosa*, could communicate directly

59 with plant host and subvert plant innate immunity by inducing plant sterol production and thereby,

60 attenuating receptor signalling through hindering the receptor clustering and plant endocytosis. The

61 results significantly enrich our understanding of the mechanisms in the tug-of-war between bacterial

62 pathogenesis and host immunity.

63

64 **INTRODUCTION**

65   Bacteria use quorum sensing (QS) to precisely coordinate population behaviors in response to

66 various environmental cues. QS signals are small molecules that contribute to bacterial virulence in

67 bacterial-host interactions by either regulating bacterial type III secretion or priming host immune systems

68 (1-3). Although the contribution of quorum sensing signals to bacterial pathogenicity has been examined

69 extensively, the direct effects of these small molecules on host biology remain under-explored.

70   QS signals produced by human pathogens, such as N-3-oxododecanoyl homoserine lactone

71 (3OC12-HSL), modulate pathogen-associated molecular patterns (PAMPs)-mediated NF-kB signaling in

72 macrophages and innate immune responses in recognition of LPS — thereby promoting persistent

73   infection (4). The 3OC12-HSL was also shown to be recognized by the bitter taste receptor, T2R family

74   protein, T2R38 in neutrophils, lymphocytes and monocytes to activate innate immune responses (5).

75   Similarly, plant pathogenic bacteria also secrete small-molecule virulence factors to cross-talk with host

76   and manipulate host immunity during infection. QS molecules produced by several bacterial

77   phytopathogens showed a wide range of interference in host development and defence mechanisms,

78   including the root morphological development in an auxin-dependent manner or callose deposition during

79   immune responses (6-8). However, the detailed mechanisms of how QS molecules directly influence

80   plant host development and pathology, especially the PAMP-mediated host immunity, remains elusive.

81   Here, we studied the host-pathogen communication between host *A. thaliana* and a specific QS

82   molecule, the diffusible signal factor (DSF). The DSF QS molecule family is produced by diverse Gram-

83   negative bacteria, many of which are devastating phytopathogens such as *Xanthomonas campestris* pv.

84   *campestris (Xcc)*, *Xanthomonas oryzae* pv. *oryzae*, *Xylella fastidiosa*, as well as several human

85   pathogens, including *Pseudomonas aeruginosa, Stenotrophomonas maltophilia*, and *Burkholderia*

86   *cenocepacia* (9). Notably, *Xanthomonas campestris pv. campestris (Xcc)*, a global-threat phytopathogen

87   that causes black rot on crucifers (10), produces up to four different analogs of DSF (*cis*-2 unsaturated

88   fatty acids), with DSF(*cis*-11-methyl-dodecenoic acid) being the primary compound (>75%) at

89   concentrations of 40-100 μM during infection (11). DSF regulates the expression of *Xcc* Type III secretion

90   system, host cell-wall macerating enzymes and other traits *in Xcc* (9, 12), and elicited host immune

91   response associated with an enhanced *Arabidopsis PR-1* gene expression (11). However, the

92   mechanism by which this QS molecule modulates plant defense responses is mostly unknown.

93   In this study, we investigated the mechanisms by which DSF alters host physiology and

94   pathology. We demonstrated that DSF induces multiple macro- and microscopic changes in *A. thaliana*

95   cell biology and development, including root morphogenesis and plant lipid profiles. The DSF-induced

96   phytosterols production that impaired both clathrin-mediated-endocytosis (CME) for internalizing FLS2

97   and FLS2 nano-clustering on the cell surface, which therefore desensitized *Arabidopsis* immune

98   responses to the bacterial flagellum. Removing the DSF-induced sterol accumulation in *Arabidopsis* could

99   reverse the defects in host endocytosis, development, and immunity caused by DSF. However, the FLS2-

100   BAK1 interaction and MAPK activation by flg22 elicitation were unchanged, indicating uncoupled PTI

101   responses underlying DSF immune-signalling. Our studies have significant implications for the

102   understanding of bacterial QS molecule DSF function in host biology and host PAMP-triggered immunity

103   (PTI) pathways.

104

105   **RESULTS**

106   **DSF suppresses bacterial flagellin-triggered innate immune responses of *Arabidopsis*.**

107   Plants have developed multiple sophisticated defense mechanisms to recognize and respond to a

108   wide range of bacterial virulence factors. Moreover, bacterial pathogens strategically instigate or subvert

109   the host immune response by interfering with PAMP recognition (13, 14). We asked whether DSF, a

110   recently discovered QS signal produced by diverse Gram-negative pathogens (15), could dysregulate

111   pattern-triggered immunity (PTI) responses. We first examined *Arabidopsis* growth in response to DSF

112   molecules within the pathological concentration of DSF during infection (11) and found that DSF inhibited

113   primary root growth of *A. thaliana* in a dose-dependent manner without apparent effects on seed

114   germination (Fig. S1A). To test whether DSF influences flagellin-triggered immunity, we tested several

115   flagellin-triggered *Arabidopsis* responses in plants treated with 25 μM of DSF, the concentration at which

116   root growth was inhibited by 40%. We found that flg22-induced stomatal closure (aperture reduced from 4

117   μm to 2 μm) was impaired by DSF treatment (Fig. 1A). Callose deposition, a plant physical defense

118   responses to prevent pathogens infections (16), was also significantly impaired in DSF-treated seedlings

119   (Fig. 1B) as was flg22-induced Reactive Oxygen Species (ROS) burst by half (Fig. 1C). We next

120   performed a plant infection assay, in which *Arabidopsis* plants pre-treated with DSF or DMSO (mock)

121   were inoculated with *Pseudomonas syringae pv. tomato* DC3000 (*Pst* DC3000). The use of a non-DSF-

122   producing bacterium here (*Pst* DC3000) was to differentiate the direct effect of DSF on plant immunity vs.

123   the effect of DSF through the activation of the bacterial Type III secretion system and other traits

124   regulated by QS, which would otherwise be difficult to dissect as DSF-deficient or overproducing mutants

125   are less virulent on plants. In this assay which involved using live bacteria with all the potential PAMPs,

126   we found that DSF-treated plants were colonized by a higher number of *Pst* DC3000 cells compared to

127   control plants, indicating a higher susceptibility of plants to *Pst* infection in the presence of DSF (Fig. 1D).

128   We further examine the ability of this QS molecule in compromising the defense mechanism of the plants

129   that were pre-protected by PTI-signaling. In treatments where plants were primed with flg22 peptide

130   before bacterial inoculation, we found that DSF application prior to flg22 priming significantly lowered

131   flg22-induced protection against infection, reflected in a higher bacterial population in DSF+flg22

132   treatment compared to that of DMSO+flg22 treatment (Fig. 1D-E).

133   **DSF interferes with the host endocytic internalization on the plasma membrane without**

134   **compromising MAPK signaling**

135   We next sought to investigate if DSF could interfere with the dynamics and functions of the

136   *Arabidopsis* flagellin receptor FLS2 (16). Flagellin binding leads to conformational changes in the FLS2

137   receptor and triggers its oligomerization (17) and endocytic internalization to activate the innate immunity

138   cascade (18, 19).

139   We first tested whether DSF affects FLS2 endocytosis upon elicitation with its corresponding

140   ligand flg22. Upon treatment of flagellin peptide flg22 but not flgII-28, FLS2 endocytic internalisation was

141   evidenced by the appearance of punctate endosomes after around 60-75 min and replenishment of

142   FLS2-GFP back to the plasma membrane (PM) at 120 min post elicitation (Fig. 2A and Fig. S1B) (18, 20,

143   21). In contrast, when FLS2-GFP seedlings were treated with DSF for 24 h prior to flg22 exposure, we

144   observed an apparent delay in FLS2 internalization upon PAMP elicitation. After 60 mins of flg22

145   elicitation, DSF-treated plants showed fewer FLS2-positive endosomes compared to DMSO-treated

146   plants, indicating the attenuated endocytosis of FLS2. We then asked whether the attenuated FLS2

147   endocytosis could also lead to a delay in FLS2 degradation (22, 23). Consistent with a previous

148   observation (24), we found that FLS2 degradation occurred approximately 30 min after peptide elicitation

149   and this response was similarly delayed in DSF pre-treated seedlings (Fig. S1C).

150   To quantitatively determine the DSF-caused defects in receptor endocytosis, we examined the

151   FLS2 endocytic internalization at the plasma membrane (PM) using variable-angle epifluorescence

152   microscopy (VAEM) (Fig. 2B). From VAEM micrographs, we quantitatively determined receptor

153   internalisation by kymograph analysis of FLS2-GFP puncta. We observed that flg22 enhanced FLS2

154   endocytosis as shown by the shortened lifetime of FLS2 on the plasma membrane, and this enhancement

155   was blocked by DSF pre-treatment prior to peptide elicitation (Fig. 2C). As FLS2 is endocytosed via

156   clathrin-mediated endocytosis (CME) (20), we then tested whether the delay in FLS2 endocytosis was

157   due to a general inhibition of CME. Indeed, we observed a significant increase in the lifetime of clathrin-

158   light-chain (Fig. S2A), as well as the lifetime of several typical CME-dependent endocytic receptor:

159   brassinosteroid receptor BRI1 and Boron receptor BOR1 under DSF treatment (Fig. S2B-C), indicating

160   the impaired endocytic internalization of these PM receptors.

161   Unexpectedly, although DSF delayed FLS2 degradation and recycling due to the impaired CME

162   (Fig. S1C), we did not observe a noticeable change in the acute response of MAPK phosphorylation,

163   which occurs approximately 15 min post-elicitation and returns to basal level within 60 min (Fig. S1D).

164   This result suggests that flg22-induced MAPK activation is independent of FLS2 endocytosis under DSF-

165   signalling, which is consistent with the unchanged *FRK1* up-regulation in the presence of flg22 (Fig. S1E)

166   that is through the activation of a MAPK signalling cascade (25). Exposure to flg22 could still induce the

167   expression of *FRK1* in DSF-treated seedlings, suggesting that DSF did not have a significant effect on

168   *FRK1*-related pathway and might not influence FLS2-BAK1 heterodimerization that was critical for *FRK1*

169   induction (26). In agreement with this observation, we also found that DSF did not affect the flg22-induced

170   FLS2-BAK1 heterodimerization (Fig. S1F), suggesting that the inhibition of flg22-induced ROS burst by

171   DSF is unlikely a direct consequence of disturbed FLS2-BAK1 complex formation, and uncoupled from

172   MAPK activation.

173   **DSF impairs lateral nano-clustering of FLS2 on the plasma membrane**

174   In addition to the endocytic internalization that removes material from the plasma membrane, the

175   lateral nano-clustering of plasma membrane-bound receptors through multivalent interactions is well-

176   known to mediate diverse signal transduction mechanisms during host-pathogen interactions (27; 28).

177   The self- or hetero-oligomerization of surface receptors are critical for receptor activation, signal

178   amplification, and signal transduction in both plants and human, such as the brassinosteroid receptor

179   BRI1 (29), chitin receptor *At*CERK1 (30), EGF receptor (31, 32) or insulin receptor IR/IGFR (33). To

180   further investigate DSF-induced effects on receptor lateral dynamics and their activity, we performed

181   living cell imaging of FLS2-GFP, chosen from several DSF hindered endocytic PM receptors (Fig. 2C, Fig.

182   S2B-C). FLS2 formed heterogeneous PM clusters with or without ligand activation (Fig. 2B), representing

183   the resting- or activated- states, respectively (34). The formation of FLS2-BAK1 heterodimer upon flg22

184   elicitation is a key step in activating downstream defense signaling (35, 36). Besides the FLS2-BAK1

185   interaction, an increase in FLS2-FLS2 self-oligomerization is also important for FLS2 phosphorylation and

186   signal transduction (37). Though DSF did not influence flg22-triggered FLS2-BAK1 interaction and MAPK

187   activation (Fig. S1D,F), the fact that DSF impaired the ROS production suggested uncoupled

188   mechanisms in the activation of MAPK and the ROS production by potential using differential inter- or

189   intra-protein interactions of FLS2 for these processes. Consistently, with a DSF pre-treatment, FLS2

190   clusters became more diffused with a lower spatial-clustering index (SCI) after 60 min of flg22 elicitation

191   (Fig. 2D).

192   To study the detailed physical interactions of FLS2 receptors in the multivalent nanoclusters, we

193   took advantage of a well-established living cell imaging approaches in studying mammalian receptor

194   clustering and activation (38, 39). We quantitatively measured the lateral self-interaction of FLS2 on the

195   plasma membrane directly under ligand activation by performing FRET between identical fluorophores

196   (homo-FRET) coupled with VAEM, which allows sensitive measurement of fast cargo-induced receptor

197   activation (40). In this method, homo-oligomerization of FLS2-GFP will be accompanied by a reduction of

198   fluorescence emission anisotropy of FSL2-GFP when it is excited by polarized light. We determined the

199   steady-state fluorescence emission fluorescence anisotropy of FLS2-GFP with or without 5 minutes of

200   peptide elicitation (Fig. 2E, Fig. S2D,E). Flg22 stimulated a significant decrease in anisotropy of FLS2-

201   GFP, reflecting the flg22-induced oligomerization of FLS2. However, DSF-treated plants showed a

202   reduction of flg22-triggered anisotropy change, indicating a suppressed formation of homo-oligomer

203   (active) from homo-dimer states (unstimulated) (41). Seedlings exposed to DSF did not exhibit any

204   significant change in emission anisotropy of FLS2 after flg22 elicitation (compared to DMSO+flg22

205   control, $p > 0.05$) (Fig. 2E), consistent with SCI analysis (Fig. 2D).

206   We next investigated the effects of DSF on bulk endocytosis using the lipophilic dye FM4-64 and

207   CME using transgenic plant expressing PIN2-GFP (42). Briefly, we monitored the uptake of FM4-64 in

208   *Arabidopsis* seedlings expressing PIN2-GFP, an auxin efflux carrier protein and a CME-dependent cargo

209   (43) in the presence of the novel small-molecule inhibitor ES9-17, which specifically binds to clathrin

210   heavy-chain without protonophoric effect (44, 45). To test whether DSF would influence CME further on

211   top of a partially-compromised CME by small molecular inhibitors (46), we pre-treated PIN2-GFP

212   seedlings with DMSO/DSF and then, performed pharmacological studies with different combinations of

213   ES9-17/BFA on treated plants, using FM4-64 dye as an indicator of bulk endocytosis. Our result indicated

214   that while neither DSF nor 50 µM ES9-17 completely blocked the internalization of FM4-64 dye, a

215   combined treatment of DSF and ES9-17 totally inhibited FM4-64 uptake, indicated by the absence of

216   intracellular FM4-64 signal and FM4-64-containing BFA bodies in BFA treatment (Fig. 3A-D). Our results

217   demonstrated the additional attenuation of bulk-phase endocytosis by DSF on top of the CME inhibitor

218   ES9-17.

219   We then asked whether such impairment of plant CME could also disrupt endosomal transport

220   after endocytic invagination from the plasma membrane. We examined the intracellular membrane

221   compartments of the protein sorting and endosomal transport pathways upon DSF treatment. No

222   noticeable ultrastructural changes were observed in early endosome/trans-Golgi network (VHA-a1), late

223   endosome/multivesicular body (MVB) (RabF2b), and Golgi (Got1p homolog), nor in their response to

224   Brefeldin A (BFA) or Wortmannin that induce Golgi aggregation or MVB dilation (47), respectively (Fig.

225   S3A-D). The above results suggest that DSF likely impairs plant endocytosis mainly at the step of

226   endocytic internalization on the plasma membrane and not at the endomembrane trafficking steps.

227

228   **DSF increased host phytosterol contents and reprogramed *Arabidopsis* lipid metabolism**

229   Dynamic behaviors of PM proteins, including the endocytic invagination, lateral diffusion, and

230   inter-molecular interactions, rely on the lipid-compartmentalization and a balanced sterol composition of

231   the plasma membrane (39, 48). We next sought to determine if the lipid profile of *Arabidopsis* was also

232   altered by DSF, given that this QS signal is a short-chain fatty acid-like molecule that could serve as a

233   building block to create diverse biologically active molecules (2). Our targeted lipidomic profiling of

234   *Arabidopsis* seedlings revealed that *Arabidopsis* seedlings grown on DSF-supplemented medium had a

235   comprehensive shift in a wide range of lipid compounds compared to those grown on control medium

236   (Fig. 3E-F; Dataset S1-2). Interestingly, the most notable changes were observed in several acyl steryl

237   glycosides (ASGs), of which the most abundant phytosterols: sitosterol, campesterol, and stigmasterol

238   showed an increase by 2-4 folds. Sterols regulate a wide range of cellular processes, such as lipid

239   metabolism and cell membrane dynamics, such as the formation of membrane microdomains (49, 50).

240   We, therefore, tested whether the increased sterol content could explain the DSF-induced phenotypes by

241   treating *Arabidopsis* seedlings with Methyl-β-cyclodextrin (MβCD) in combination with DSF. MβCD is a

242   widely-used sterol-depleting compound that acts strictly at the membrane surface by binding to sterol with

243   high-affinity and remove sterol acutely within minutes, with demonstrated success in acute depletion of

244   sterols in both plant and animal cells (51-55). Here, we found that MβCD fully blocked the DSF-induced

245   inhibition of primary roots and root hairs, suggesting DSF's opposite effects compared to MβCD on sterol

246   perturbation in *Arabidopsis*. Consistent with this result, culturing seedlings in the presence of both MβCD

247   and DSF completely abolished the DSF-triggered increase of lipid components, including phytosterols.

248   The total quantity of measured sterols content in MβCD and MβCD+DSF treatment were reduced to 92.4

249    $\pm$ 10.3 nmol.g^{-1} and 76.0 $\pm$ 10.4 nmol.g^{-1} respectively, compared to 138.2 $\pm$ 24.6 nmol.g^{-1} in DMSO

250   treatment and 366.3 $\pm$ 121.1 nmol.g^{-1} in DSF treatment, showing a dominant effect of MβCD over DSF

251   (Fig. 3F).

252   To further examined the role of phytosterols in DSF-induced inhibition of primary root growth, we

253   performed growth assay on several *Arabidopsis* mutants of the sterol biosynthesis pathway (Fig. 3G) in

254   the presence of DSF. The *smt1-1* and *fk-X224* mutants were chosen for this experiment as their growth

255   defects are less severe compared to other sterol mutants. Primary root growth of *smt1-1* mutant

256   (defective in C-24 methyltransferase) and the *fackel (fk)* mutant *fk-X224* (defective in sterol C-14

257   reductase), which are two key early steps of sterol biosynthetic pathway upstream of the branch point

258   (56) (Fig. S3E), showed significantly reduced sensitivity to DSF, as represented by a lower inhibition rate

259   compared to their corresponding wild-type ecotypes (Fig. 3G, Fig. S3F). Together, these findings support

260   our hypothesis that DSF regulates host development through altering host phytosterols content.

261   **Sterols regulation of lipid microdomain assembly and endocytosis phenocopies DSF function in**

262   *Arabidopsis*

263   As we observed that MβCD and DSF have opposite effects on *Arabidopsis* lipidomic profiles in

264   the long-term plant growth assay, we next examined whether an acute sterol removal by MβCD would

265   restore other DSF-induced phenotypes on plant endocytosis and innate immune responses of

266   *Arabidopsis*. In the experiments testing DSF perturbation of PTI, following MβCD pre-treatment, the

267   number of FLS2-positive endosomes in FLS2-GFP plants pre-treated with DSF increased significantly at

268   60 min after elicitation with flg22, even though MβCD itself strongly inhibited FLS2 trafficking into

269   endosomes (Fig. 4A). In line with this finding, MβCD also restored the bulk endocytosis of FM4-64 dye

270   that was blocked by the combined treatment of DSF and ES9-17, represented by the similar ratio of

271   intracellular/PM signal intensity and BFA bodies area between DMSO and ES9-17 treatment in the

272   DSF+MβCD pre-treated seedlings (Fig. 4B and Fig. S4A), the delay of CLC lifetime (Fig. S4B), and the

273   DSF-induced inhibition of ROS burst (Fig. 4C).

274   We further asked whether the supplementation of phytosterols could phenocopy the DSF-induced

275   defects in CME and flg22-activated FLS2 internalization. We found that exogenous phytosterol mix

276   (Sitosterol: Stigmasterol: Campesterol=8:1:1) treatment phenocopied DSF-induced inhibition of flg22-

277   stimulated ROS production and subsequent MβCD supplement reversed the sterol-inhibited endocytosis

278   of FM4-64 dye in the presence of ES9-17 (Fig. S4C-D), FLS2 internalization into endosomes (Fig. S5A),

279   as well as flg22-triggered ROS burst (Fig. S5B). Similar to the sterol depleting compound MβCD, we

280   observed that Lovastatin— a sterol biosynthesis inhibitor— also rescued ROS burst in DSF-treated

281   leaves (Fig. S5C), suggesting that the DSF-induced inhibition of ROS and innate immunity was likely due

282   to the incorporation of DSF into phytosterol metabolic pathways or induction of sterol production via an

283   unknown mechanism. Similar inhibitions of plant root growth were also observed with the predominant

284   sterol β-sitosterol: β-Sitosterol could also inhibit root growth in a dose-dependent manner similar to DSF,

285   and this inhibition could also be reverted by MβCD treatment (Fig. S5D-E). Together, these data suggest

286   that a balance in sterol composition of the plant PM is crucial for plant CME as well as the pattern-

287   recognition receptors (PRRs)-mediated defense responses.

288   **DSF and Sterol interfere with lateral membrane compartmentalization and FLS2-nanoclustering**

289   Lipids composition, compartmentalization, and surface tension of plasma membrane are critical

290   physicochemical properties in regulating lateral motility, protein-protein interaction, and distribution of

291   immune receptors, including FLS2 (28, 57). Sterols are essential constituents of the liquid-ordered (L_o)

292   lipid nanodomains and influence membrane flexibility (58). We, therefore, asked whether the DSF-

293   induced phytosterol increase influences the compartmentalization of PM by imaging nanodomain-marker

294   YFP:REM1.2 *Arabidopsis* expressing YFP:REM1.2 treated with DSF, a phytosterol mix (sitosterol:

295   stigmasterol: campesterol=8:1:1) or MβCD were examined via super-resolution 2D-Structured Illumination

296   Microscopy (2D-SIM). MβCD resulted in a significant reduction of the mean intensity of YFP:REM1.2-

297   marked microdomains, suggesting a reduction in the ordered-phase. The presence of either DSF or

298   exogenous sterol could similarly reverse the MβCD effect in lipid compartmentalization (Fig. 5A-B).

299   Furthermore, with MβCD post-treatment, DSF no longer attenuated the homo-oligomerisation of FLS2

300   upon flg22-elicitation, indicated by an anisotropy reduction in living cell homo-FRET imaging (Fig. 5C-D).

Osmotic stress rescued DSF-induced inhibition of *Arabidopsis* endocytosis

302   Lipid order increase and microdomain assembly deform the organization of local plasma

303   membrane and thus, alter the line tension of phase-separated PM, which are physical cues in regulating

304   endocytosis and lateral molecular dynamics (48, 59, 60). We were therefore motivated to ask whether

305   osmotic stress, which induces plant PM remodelling (48), could reverse the inhibition of endocytosis

306   caused by DSF. To test if osmotic stress could restore the inhibition of endocytosis and plant growth

307   caused by DSF, we measured primary root length from seedlings grown in combination with different

308   concentrations of osmolytes (NaCl or Mannitol) and DMSO/DSF. We observed a partial recovery of DSF-

309   induced inhibition on primary root growth at 10 and 50 mM NaCl, but not with Mannitol (Fig. S6A).

310   Consistent with this result, flg22-induced internalization of the FLS2 receptor in seedlings, which were

311   incubated with 100 mM NaCl following DSF treatment, could also be observed as early as 30 min after

312   peptide elicitation (Fig. S6B). The differential recovery in root growth by Mannitol and NaCl treatments

313   suggested that while their alteration of PM tension reverts the DSF-attenuated endocytosis similarly, ionic

314   and non-ionic osmolytes may have different mechanisms to achieve a full reversal of root development

315   under DSF-signaling.

316

DISCUSSION

318   Hosts and symbionts constantly exchange small metabolites during interkingdom interactions. For

319   example, the plant pathogenic bacterium *Pseudomonas syringae* produces the phytotoxin coronatine to

320   target multiple plant immunity pathways, including stomatal defence, thus facilitate bacterial entry as well

321   as disease development (61-64). Conversely, the transfer of small molecules from plants to microbes is

322   also an integral part of microbial symbiotic relationships (65, 66).

323   During host-pathogen interaction, bacteria mainly rely on quorum sensing to regulate the

324   expression of virulence genes necessary for colonization and pathogenesis or to switch between different

325   lifestyles (67-69). Numerous reports highlighted the effects of QS molecules on plant development and

326   immunity, but mechanistic insights on how bacterial QS molecules specifically interfere with host biology

327   remain sparse (70). Distinctive acylation and hydroxylation patterns in bacterial metabolites have been

328   suggested as a molecular signature of AHL QS family to regulate host immune responses (71, 72). For

329   example, medium-chained 3-hydroxy fatty acids directly bind and trigger the phosphorylation of

9

330 LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE) receptor kinase, leading to

331 activation of cytoplasmic receptor-like kinases and stimulate *FRK1* expression (71, 73). Here, we

332 unravelled a different mechanism by which a unique QS molecule called DSF regulates host biology by

333 perturbing host phytosterol homeostasis. Unglycosylated sterols and glycosylated sterols (including steryl

334 glycosides and acyl sterol glycosides) are integral components of the plant plasma membranes,

335 especially in membrane microdomains (50, 74). The increase in host acyl-glycosylated phytosterols by

336 DSF treatment shown in our targeted lipidomic analysis might be a consequence of direct sterol

337 modifications – such as DSF being metabolized to a precursor of acyl source for steryl glucoside

338 acyltransferase (75), or from a potential sterol exchange between PM and ER through PM-ER contact

339 sites (76, 77) — both of which require further investigation. Through the modulation of sterols, DSF

340 interferes with plant PTI through the alteration of both the clustering and endocytic internalization of the

341 surface receptors. Compared to untreated plants, DSF-treated plants display an apparent increase in total

342 phytosterol by >2.5 fold, as well as slight enhancement in several other lipid species that are PM

343 structural constituents, such as glycerolipids (PC, PE, PG, and PS) and sphingolipids (ceramides) (78-

344 80). Phosphatidylserine (PS) is known to play an essential function in surface membrane

345 compartmentalization and protein clustering (81, 82). Thus, how the changes of phytosterols and PS by

346 DSF tune the PM mechanical properties is worth of future studies via artificial lipid bilayer-based assays

347 and membrane modelling.

348 The diverse roles of QS molecules from pathogenic bacteria in both regulating bacterial responses and

349 modulating host biology reflect a direct co-evolution between hosts and pathogens according to the Zig-

350 zag model (83). *Xcc* DSF gradually accumulates during infection and can reach substantial

351 concentrations (hundred micromolar amount) at later stages of disease (11), similar to QS molecules from

352 other bacteria (84). A previous report demonstrated that at high concentrations (e.g. 100 μM), DSF acts

353 as a hypersensitive reaction (HR) elicitor evident by cell-death response and excessive callose deposition

354 (11). Here, we found that at low concentrations (e.g. 25 μM), which is more relevant to the early stages of

355 infection, this QS signal seems to regulate a specific branch of plant PTI responses through the

356 modulation of molecular dynamics of PRRs on the PM. These findings of plant responses to low dose and

357 the high dose of DSF treatments are not necessarily conflicting to each other as they represent different

358 stages of plant immune responses over the accumulation of bacterial DSF during disease development.

359 In addition, the high-dose DSF-induced plant HR could also be masked by bacterial extracellular

360 polysaccharide during plant colonization, demonstrating the constant bidirectional interaction between

361 plant and bacteria (11). Taken together, these above results reflect the dynamic change in balancing the

362 tug-of-war over the accumulation of DSF molecules during bacterial infection. Although we found that

363 DSF did not directly alter PAMP-activated MAPK signaling, heterodimerization of FLS2-BAK1, or *FRK1*

364 expression, this QS molecule did compromise the PAMP-triggered ROS production. Such different DSF-

365 mediated host signalling demonstrates the uncoupled mechanisms of MAPK activation and ROS

366 production upon flg22-elicitation, in which ROS production was attenuated by DSF/sterol while activation

367   of MAPK cascade remained. As a result of a functional MAPK cascade, DSF could only reduce but not

368   entirely abolish the flg22-induced defense responses (Fig. 1D-E). FLS2-BAK1 may not be the only rate-

369   limiting components of the defense signaling cascade as *bak1* null mutants still retain a certain level of

370   MAPK and ROS responses (85), and FLS2-BAK1 association could be uncoupled from MAPK activation

371   as previously shown with the flg22 G/A mutant peptide (26). Similarly, in chitin-mediated PTI, AtCERK1-

372   mediated MAPK-activation and ROS production were also found to be distinctly regulated by receptor-like

373   cytoplasmic kinases (RLCK) PBL27 and BIK1, respectively (86; 87). These observations suggest

374   potential heterogeneity in receptor coupling and distinct constituents in receptor clusters that may convey

375   differential immune-response by recruiting different PTI signaling molecules. Similar scenarios have also

376   been observed in the mammalian innate immune system (88). The FLS2 binding-partners for MAPK and

377   ROS signaling have differential sensitivity to DSF/sterol-induced perturbation of PM, indicating their

378   possibly different affinity or participation in nanodomains and FLS2-nanoclusters during PRRs signaling.

379   Consistently, due to the active involvement in the high-ordered membrane domain of NADPH oxidase

380   proteins, the NADPH oxidase activity has also been reported to be highly sensitive to plant sterol changes

381   (89). The decline of ROS burst under DSF-signaling might also be through the change in signaling

382   competency of other components downstream of FLS2 and BAK1, such as BIK1, BSK1 or heterotrimeric

383   G protein subunits; or the function of the membrane-bound RBOHD itself (90; 91; 92; 93; 94). Of note, the

384   loss-of-function alleles of some of these components have also been documented to affect the flg22-

385   induced ROS burst but not MAPK activation (90; 91; 92; 95).

386   Increasing evidence supports the roles of membrane microdomains in host innate immunity by

387   regulating surface receptor dynamics in and out of microdomains on plasma membrane upon ligand-

388   recognition, subsequent receptor interactions, and innate immunity activation (27; 96; 97). For instance,

389   disruption of PM continuity is competent enough to abolish the signaling carried out by multiple PM

390   receptors (BRI1, FLS2, EGFR) (28, 98). Similar to many other immune-receptors (99), unstimulated

391   *Arabidopsis* FLS2 could be in a dimer form (41). Our living cell homo-FRET approach allowed us to

392   quantitatively measure the dynamic formation of the nanoclusters of plant surface molecules (39). Homo-

393   FRET experiments revealed that flagellin triggered the nano-clustering of FLS2 from a less to more

394   oligomerized, representing a transition from the unstimulated to active state during early immune

395   responses.

396   Our data suggest a plausible model (Fig. 6) in which the *Xcc* QS molecule DSF alters sterols

397   composition to modulate membrane microdomains and thereby, affects both the general endocytosis

398   pathway as well as FLS2 receptor nano-clustering upon PAMP stimulation, and thus the ROS production

399   (100). Sterols, together with sphingolipids, are major constituents of plasma membrane lipid

400   raft/microdomains that are formed by diverse mechanisms of molecular assemblies of surface molecules

401   (100-102). Distinct microdomains spatially separate from each other and their assembly and distributions

402   are of significant importance for the regulation of diverse cellular processes, including the nano-clustering

403   or CME of surface signaling factors (41, 103, 104). In agreement with this model and our data, sterol

404 content has previously been found to directly or indirectly influence membrane organization and CME (41,

405 58, 104, 105). CME is well-known to coordinate PTI signaling in plants 106. However, as ligand-induced

406 MAPK activation (~5-30 min post elicitation) is detected earlier than the appearance of endocytosis-

407 mediated PRR-accumulation in endosomes (~30 min-1 h post elicitation) (18, 26) (Fig. 2A), endocytic

408 invagination per se might not be a direct mediator for MAPK activation (24), which is also supported by

409 the fact here that an attenuated-endocytosis by DSF did not alter flg22-mediated MAPK activation.

410 Nevertheless, the impairment of endocytosis could still influence the internalization and recycling of the

411 surface PRRs at different oligomerization states upon PAMP-elicitation (24, 107). Consistent with this

412 notion, recent work also highlighted many consequences of the CME impairment in the *Arabidopsis*

413 clathrin mutant *chc2*, such as FLS2 endocytosis, stomatal defense, callose deposition, as well as

414 increased susceptibility to bacterial infection, while acute MAPK response remained intact (20).

415 During bacterial propagation from early- to late- stages of infection, DSF attenuates the

416 continuous enhancement of the host PTI responses in ROS production that could otherwise be stimulated

417 by the increasing amount of PAMPs. Receptor activities in both lateral motility for nano-clustering or

418 internalization for receptor recycling on the plasma membrane are compromised by DSF signalling. Our

419 report emphasizes the role of QS molecules beyond intraspecies communication, which has important

420 implications for plant disease control (3). It will also be of particular interest to investigate if the regulation

421 of host metabolic pathways by different small molecules from pathogens is a ubiquitous phenomenon

422 during host-pathogen interaction. In addition, since quorum-sensing molecules from human pathogens,

423 such as *P. aeruginosa* have also been documented to disrupt host membrane microdomains (72), it is

424 worth future studies to investigate whether signalling of plasma membrane receptors such as TLR5 (an

425 ortholog of FLS2) is also affected by such molecule in a sterol-dependent manner.

426

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442

443   **Contributions**

444   T.M.T., Z.M., and Y.M. conceived and designed the study. T.M.T. and Z.M. performed most of the

445   plant-based experiments. T.M.T., A.T., and F.T. performed lipidomics analysis and interpreted the data.

446   Z.M. and S.N. performed homo-FRET based imaging, image analysis, and data interpretation. Y.C helped

447   with the quantitative RT-PCR and data analysis. X.H. and J.W. performed the EM works. B.G. and J.L.

448   performed the CoIP experiment. F.T., M.R., S.M., J.L., and L.Y. provided essential intellectual input and

449   data interpretation. T.M.T. and Y.M. wrote the manuscript with feedback from all authors. Y.M. supervised

450   the study.

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731

FIGURE LEGENDS

Fig. 1. Diffusible-signal factor (DSF) simultaneously dampens several plant defense responses in *Arabidopsis thaliana* against the bacterial PAMP flg22. (A) Stomatal apertures of 5-week old Col-0 leaves after flg22 elicitation. Intact leaves were infiltrated with DMSO or 25 μM DSF diluted in 10 mM $MgCl_2$ for 24 h before the epidermal layers of detached leaves were peeled off and treated with 1 μM flg22, stained with propidium iodine and imaged. Stomatal apertures were measure by Fiji (n$\geq$130 stomata, bars, 10 μm). **(B)** Callose deposition in 2-week old Col-0 plants pre-treated with DMSO or 25 μM DSF in ½ liquid MS for 24 h before being elicited by flg22 in liquid ½ MS. Leaves were stained with aniline blue and imaged using a confocal microscope with UV excitation to visualize flg22-triggered callose deposition. Plants treated with only DMSO or DSF were used as negative control (n$\geq$40 images; bar, 100 μm) **(C)** Reactive oxygen burst in Col-0 leave strips pre-treated with DMSO or 25 μM DSF for 24 h prior to flg22 elicitation (n$\geq$6 leaf discs/condition). The experiment were repeated three times. **(D)** Bacterial population at 4 days post-inoculation (DPI) in *Arabidopsis* 2-week old seedlings pre-treated with DMSO (solvent control) or DSF, followed by priming with flg22 peptide and flooding assay with *Pst* DC3000. Boxplot represent pooled data from two independent experiments (n=21). **(E)** Protection index (%) was derived from log CFU/mg tissue by dividing the differences between flg22-treated group and the control group to the mean bacterial population of the control group. P-values were determined by one-way ANOVA (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant).

Fig 2. DSF delays ligand-induced endocytosis of the FLS2 receptor in response to flg22 peptide.
(A) Five-day old FLS2-GFP seedlings were treated for 24 h in liquid 1/2 MS medium supplemented with 25 μM DSF or DMSO (solvent control) before being elicited with 10 μM flg22 and imaged at indicated timepoints. Micrographs represent maximum projections of 12 slices every 1 μm z-distance (bars, 20 μm). Bar graphs represent the numbers of endosomes per image. Error bars, SEM (n$\geq$12) **(B)** FLS2-GFP receptors on the PM of *Arabidopsis* cotyledons. Five-day old FLS2-GFP plants were treated with DMSO or DSF for 24 h prior to elicitation by 10 μM flg22 and imaged using VA-TIRFM (bar, 1μm). **(C)** Kymographs of FLS2-GFP clusters on *Arabidopsis* plasma membrane before and after 60 min of flg22 elicitation, following DMSO or 25 μM DSF 24-h pre-treatment. The lifetime of FLS2-GFP foci was measured from kymographs using Fiji (n$\geq$ 100 endocytic events). **(D)** Boxplot represents spatial clustering of FLS2 receptor foci, indicated by Spatial Clustering Index (SCI). SCI was calculated from TIRF timelapse movies based on the ratio of top 5% of pixels with the highest intensity and 5% of pixels with the lowest intensity (n$\geq$60 ROIs). **(E)** Homo-FRET analysis of receptor oligomerization on PM of DMSO- and DSF-treated FLS2-GFP seedlings upon flg22 elicitation for 5 min. As the plasma membrane is not definite flat, anisotropy quantification was performed only in those well-focused regions of the plasma membrane (white dashed lines) (bar, 5 μm). Boxplot represents anisotropy values calculated from homo-FRET imaging of FLS2-GFP. Representative intensity and anisotropy images were shown. Insets are

769   representative 20x20 pixel ROIs used for data analysis (n≥150 ROIs). P-values were determined by one-

770   way ANOVA (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns, not significant).

771

772   **Fig. 3. DSF inhibited endocytosis in *Arabidopsis* roots by altering plant lipid profile. (A)**

773   Internalization of the endocytic tracer FM4-64 in PIN2-GFP seedling roots pre-treated with DMSO or DSF

774   for 24 h. Plants were treated in liquid ½ MS with DMSO (control) or 50 μM ES9-17 for 1 h before being

775   stained with FM4-64 and visualised using confocal microscopy (n≥5 plants; bars, 10 μm). **(B)**

776   Accumulation of intracellular FM4-64 signals into BFA compartments after BFA treatment. PIN2-GFP

777   seedlings pre-treated for 24 h on ½ MS agar supplemented with DMSO or DSF were subjected to BFA or

778   ES9-17+BFA treatments before being stained with FM4-64 and imaged (n≥5 plants; bars, 10 μm). (**C**)

779   Boxplot represents the ratio of intracellular/plasma membrane signal intensity of FM4-64 dye in root cells

780   presented in Fig. 3A as an indicator of bulk endocytosis (n≥37 cells). (**D**) Boxplot represents the area of

781   BFA compartments in seedlings grown on DMSO- or DSF-supplemented plates as presented in Fig. **3B**,

782   n≥161 BFA compartments. **(E)** Growth of Col-0 seedlings on 1/2 MS agar supplemented with DMSO, 25

783   μM DSF, 2mM MβCD and 25 μM DSF+2mM MβCD. Images of whole seedlings and root hairs were taken

784   after seven days (top panel bars, 10 mm; bottom panel bar, 1mm). The length of primary roots (n≥20) and

785   root hairs (n≥140) of 7-day old seedlings on different medium measured by Fiji. **(F)** Lipidomic profiles of

786   *Arabidopsis* Col-0 seedlings after seven days growing on 1/2 MS agar supplemented with either 25 μM

787   DSF, 2 mM MβCD or 25 μM DSF + 2 mM MβCD. Heatmap representing the log2 value of fold change

788   compared to DMSO treatment (n=6 replicates). The bar graph parallel to the heatmap indicates the

789   relative abundance (in percentages) of the corresponding species within each lipid class. **(G)** DSF-

790   induced Inhibition rate of sterol mutants *fk-X224* and *smt1-1* and their corresponding wildtype ecotypes

791   Ler and Ws. Seedlings were monitored for seven days on DMSO- and 25 μM DSF- supplemented ½ MS

792   plates (n≥28 plants). Inhibition rate (%) was represented by the ratio between the difference in primary

793   root length of seedlings on control (DMSO) vs. DSF-supplemented medium and the mean primary root

794   length of control seedlings. P-values were determined by one-way ANOVA (* p < 0.05; ** p < 0.01; *** p <

795   0.001; **** p < 0.0001; ns, not significant).

796

797   **Fig. 4. Sterol depletion by MβCD recovered DSF/sterol-induced phenotypes on *Arabidopsis***

798   **seedlings.**

799   **(A)** FLS2 receptor internalization into endosomes monitored by confocal microscopy following 10 μM

800   flg22 treatment. FLS2-GFP seedlings were pre-treated for 24 h with DMSO or 25 μM DSF in ½ MS,

801   followed by a brief 2 mM MβCD treatment for 30 min prior to peptide elicitation. Micrographs represent

802   maximum projections from 12 slices taken every 1 μm z-distance. Bar graphs showed the number of

803   endosomes per image (n≥10 images; bar, 20 μm). (**B**) Internalization of FM4-64 dye of PIN2-GFP

804   seedling roots after plants were treated for 24 h on ½ MS plates with 25 μM DSF followed by 30 min

805   treatment with 2 mM MβCD in liquid ½ MS. Plants were treated with 50 μM ES9-17 for 1 h and/or 50 μM

806   BFA-1h before staining with FM4-64, and DMSO was used as solvent control (n$\geq$5 plants; bar, 10 μm).

807   Quantification of intracellular/PM ratio were performed on n$\geq$50 cells and BFA bodies area measurement

808   were performed on n$\geq$250 BFA bodies. **(C)** ROS production by Col-0 leaf strips treated for 24 h with

809   DMSO or 25 μM DSF, followed by a 30-min treatment with water (control) or 2 mM MβCD prior to

810   elicitation by 1 μM flg22 peptide. The experiment were repeated twice with similar result. P-values were

811   determined by 2-tailed Student's *t*-test (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns, not

812   significant).

813

814   **Fig. 5. Lipid microdomain integrity is dependent on proper sterol composition of the PM. (A)** 2D-

815   SIM images of the microdomain marker REM1.2-GFP taken after 24 h treatment with DMSO, DSF, or

816   sterol mix followed by a 30 min treatment with 2 mM MβCD or water control (bar, 2 μm). **(B)**

817   Quantification of Remorin foci intensity on PM (n=500 foci). **(C)** Oligomerisation state of FLS2-GFP

818   receptors before and after 5 min of elicitation with 10 μM flg22 following treatment of the seedlings MβCD

819   or DSF+ MβCD. Representative intensity and anisotropy images were shown. Insets are representative

820   20x20-pixel ROIs used for data analysis (bar, 5 μm). **(D)** Boxplot represents anisotropy values measured

821   from Homo-FRET experiment (n$\geq$150 ROIs). P-values were determined by one-way ANOVA (* p < 0.05; **

822   p < 0.01; *** p < 0.001; **** p < 0.0001; ns, not significant).

823

824   **Fig. 6. Proposed model for DSF-induced suppression of plant innate immunity by pertubation of**

825   **PM lipids.** Upon contact with bacteria/flagellin, FLS2 receptors form homodimers and heterodimers with

826   interacting partners on the plasma membrane. Activated receptors then relay the signals to PM co-

827   receptors and cytoplasmic kinases to induce a wide range of defense responses and also undergo

828   endocytosis. However, in plants infected with DSF-producing bacteria, DSF could be metabolized into

829   plant sterols or directly induce the production of phytosterols via unknown mechanisms. Sterol enrichment

830   on the plant PM leads to an increase in lipid ordered phase - L_o (or PM microdomains) and a decrease in

831   lipid disordered phase (L_d phase). Receptor nanoclustering were affected and as a result, they could not

832   oligomerize and undergo endocytosis properly. Therefore, signalling cascades from plasma membrane

833   that result in PTI responses were attenuated.

834

835 **Fig. S1. DSF affected plant growth and flagellin receptor degradation (A)** Growth of *Arabidopsis* Col-

836 0 seedlings seven days on 1/2 Murashige-Skoog agar supplemented with different concentrations of DSF

837 (bar, 10 mm, n≥14). **(B)** Confocal micrographs of the FLS2 receptor signal on PM of FLS2-GFP seedlings

838 after treatment with 10 μM flgII-28 (bar, 10 μm). Micrographs are maximum projections of 12 slices at

839 every 1 μm z-distance. Bargraph on the right represent number of endosomes per image quantified from

840 maximum-projected images (Error bars, SEM, n≥12). **(C)** Degradation of FLS2 receptor was delayed in

841 DSF-treated plants. Leaf strips of 5-week old FLS2-GFP plants were floated for 24 h in water containing

842 DMSO or 25 μM DSF, then elicited with 10 μM flg22 and snap-frozen in liquid nitrogen at indicated

843 timepoints. Total protein extracts were probed with α-GFP antibody to detect FLS2. Staining of the

844 membrane with Ponceau S served as loading control. The experiment was repeated three times with

845 similar results **(D)** Western blot analysis of MAPK responses of Col-0 leaf strips in response to the flg22

846 peptide at different time-points, following 24 h treatment in 1/2 MS supplemented with DMSO or 25 μM

847 DSF. Ponceau S staining of the WB membrane was shown as loading control. The experiment was

848 repeated twice with similar result. **(E**) Relative transcript abundance of *FRK1* as measured by quantitative

849 RT-PCR of *FRK1* expression. Data represent relative expression to control calculated using the ΔΔCt

850 method. Columns represent mean relative gene expression level of at least six technical replicates from

851 two biological replicates (Error bars, SEM). **(F**) Co-immunoprecipitation assay on *Arabidopsis* protoplast

852 co-transfected with FLS2-HA and BAK1-FLAG constructs. Transfected protoplasts were treated with 1 μM

853 DSF for 12 h and elicited with 1 μM flg22 for 10 min and CoIP was performed with anti-FLAG antibody.

854 The experiment was repeated twice with similar result. P-values were determined by one-way ANOVA ([*]

855 $p < 0.05$; [**] $p < 0.01$; [***] $p < 0.001$; [****] $p < 0.0001$; ns, not significant).

856 **Fig. S2. Receptor endocytosis and clustering is affected by DSF**

857 **(A-C)** Kymographs and lifetime of clathrin light chain (CLC-GFP, n≥250 events), brassinosteroid receptor

858 BRI1 (BRI1-GFP, n≥136 events) and Boron receptor BOR1 (BOR1-GFP, n≥198 events). Kymographs

859 were derived from VA-TIRF images obtained from corresponding marker lines grown on ½ MS plates for

860 5-6 days, then treated with DMSO (control) or 25 μM DSF for 24 h in ½ MS. **(D)** Anisotropy measured for

861 purified GFP protein solution at definitive TIRF (incident angle was set as 1880), and two HILO angles

862 (1910 and 2000). Quantification data shows similar anisotropy values can be obtained in these three

863 different incident angles (bar, 2 μm, n≥42 ROIs). **(E)** Anisotropy measured at HILO-TIRF gave the same

864 result as definitive TIRF, in which samples could be illuminated evenly. Anisotropy of control and MβCD

865 (10 mM in Glucose M1 buffer for 45 min at 37°C) treated cells were measured at definitive TIRF (1880)

866 and HILO-TIRF (1950 and 2000) using stably transfected GFP-GPI construct in CHO cells.

867 Measurements show the same results at definitive TIRF (1880) and HILO-TIRF (1950); and at HILO-TIRF

868 (2000) similar values can be obtained if measurements were considered from evenly illuminated area,

869   which is highlighted by dotted line (bar, 5 μm, n$\geq$30 ROIs). P-values were determined by one-way ANOVA

870   (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns, not significant).

871   **Fig. S3**. **DSF did not cause morphological changes in *Arabidopsis* endosomal compartments. (A-**

872   **C)** Confocal micrographs of DMSO/DSF-pretreated *Arabidopsis* early endosome marker line (VHAa1-

873   GFP), late endosome marker line (RabF2b-YFP), and Golgi marker line (Got1p-YFP) stained with FM4-64

874   dye with or without BFA treatment (bar, 10 μm). The relative size of enlarged multivesicular bodies (MVB)

875   and BFA compartments were measured by Fiji. **(D)** Ultrastructural observation of *Arabidopsis* organelles

876   in eight-day-old seedlings pre-treated with DMSO/DSF observed by Transmission Electron Microscopy

877   (upper panel scale bar, 200 nm; lower panel scale bar, 500 nm). Annotations on the electron-micrographs

878   are as followed: G, Golgi; TGN, trans-Golgi network; CW, cell wall; m, mitochondria; MVB, multi-vesicular

879   body; C, Chloroplast. **(E)** Sterol biosynthetic pathways in *Arabidopsis thaliana*. Corresponding mutants

880   were labelled in each step of the pathways, with dashed arrows indicating multiple biosynthetic steps. **(F)**

881   Growth of sterol biosynthesis mutants *fk-X224 and smt1-1* and their corresponding wildtype ecotypes (Ler

882   and Ws, respectively) on ½ MS agar supplemented with DMSO or 25 μM DSF. Plates were scanned after

883   seven days after sowing the seeds (bar, 5mm). The experiment was repeated twice with similar result. P-

884   values were determined by 2-tailed Student's *t*-test (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001;

885   ns, not significant).

886

887   **Fig. S4. Sterol removal by MβCD restored phytosterol-induced inhibition of endocytosis. (A)** FM4-

888   64 dye uptake by PIN2-GFP roots treated with MβCD in combination with ES9-17/BFA (n$\geq$5; bars, 10 μm)

889   **(B)** Life time of CLC-GFP in the presence of DSF, MβCD and a combination of both drugs. CLC-GFP

890   seedlings were treated with DMSO/25 μM DSF for 24 h in ½ MS medium, then subjected to a brief

891   treatment of 2 mM MβCD before being imaged using VA-TIRF. **(C-D)** Sterol mix similarly inhibits FM4-64

892   uptake in combination with the clathrin inhibitor ES9-17. Five-day old PIN2-GFP seedlings were

893   transferred to ½ MS plates containing 50 μM sterol mix **(C)** or 50 μM sterol mix + 10 mM MβCD **(D)** for an

894   additional day. Plants were subjected to drug treatments (50 μM ES9-17–1h, 50 μM BFA – 1h, or a

895   combination of both drugs) before being stained with FM4-64 and imaged (n$\geq$5, bars, 10 μm). P-values

896   were determined by one-way ANOVA (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns, not

897   significant).

898

899   **Fig. S5. Phytosterols mimic DSF-induced inhibition of receptor internalization, ROS production**

900   **and primary root growth**

901   **(A)** Internalization of the FLS2 receptor was monitored in FLS2-GFP seedling cotyledons. Plants were

902   pre-treated with 50 μM sterol mix or sterol mix + 2 mM MβCD for 24 h prior to elicitation with 10 μM of

903   flg22 and imaged at indicated timepoints. (n$\geq$12, bars, 10 μm). Boxplots represent the number of

904   endosomes per image as quantified by segmentation and particle analysis using Fiji. **(B)** ROS burst in

905 response to flg22 in Col-0 leaf strips pre-treated for 24 h with solvent control or 50 μM sterol mix, with or

906 without 2 mM MβCD. **(C)** ROS production by Col-0 leaf strips pre-treated with DMSO/25 μM DSF for 24 h

907 simultaneously with the sterol biosynthesis inhibitor Lovastatin (Lova, 1 μM). Leaf strips were then elicited

908 with 1 μM flg22 and luminescence was monitored every minute for 1 h by a BioTek Cytation 5 plate-

909 reader. The experiment were repeated twice with similar result. **(D)** Root growth of Col-0 plants on ½ MS

910 plates supplemented with different concentrations of β-Sitosterol seven days after sowing (n$\geq$22). **(E)**

911 Dose-dependent reversion of β-Sitosterol-induced growth inhibition of *Arabidopsis* Col-0 by MβCD. Col-0

912 seeds were germinated on ½ MS agar supplemented with β-Sitosterol in combination with 2 mM or 10

913 mM of MβCD. After seven days, the plates were scanned and primary root lengths were measured using

914 Fiji. Boxplot represents data from n$\geq$20 seedlings. P-values were determined by one-way ANOVA (* p <

915 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns, not significant).

916

917 **Fig. S6. Osmotic stress relieved DSF-induced inhibition of endocytosis in *Arabidopsis*. (A)** Growth

918 of Col-0 seedlings on 1/2 MS agar supplemented with DMSO or 25 μM DSF in combination with

919 increasing concentrations of NaCl or Mannitol. Seedlings primary root length was measured after 7 days

920 growing on agar plates (n$\geq$23 seedlings). **(B)** Recovery of FLS2 internalization into endosomes by NaCl.

921 Six-day old FLS2-GFP seedlings were transferred to liquid ½ MS supplemented with DMSO or 25 μM

922 DSF for 24 h, then treated with 100 mM NaCl for 1h prior to flg22 elicitation and imaged at the indicated

923 time points. Micrographs represent maximum projection of 12 slices taken every 1 μm (bar, 10 μm, n$\geq$12

924 images). P-values were determined by one-way ANOVA (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p <

925 0.0001; ns, not significant).

926

927

928 **Dataset S1.** Lipidomic profile of *Arabidopsis* Col-0 seedlings grown on ½ MS agar plates supplemented

929 with DMSO, DSF, MβCD and DSF+ MβCD. Plants were grown vertically on ½ MS agar amended with

930 appropriate chemicals in each treatment for seven days before tissues were collected for lipid extraction

931 and analysis.

932

933 **Dataset S2.** Relative fold-change of lipidomic profile of *Arabidopsis* Col-0 seedlings grown on DSF,

934 MβCD and DSF+ MβCD compared to DMSO control. P-values were determined by one-sample *t-test* to

935 determine if sample means are different than 1 at the confidence interval of 90%.

936

937 **Table S1.** Key resource table

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CONTACT FOR REAGENT AND RESOURCE SHARING

Material used in this study can be found in the Key Resource Table (Table S1). Further information and

requests for reagents and resources should be directed to and will be fulfilled by the Lead Contact,

Yansong Miao (yansongm@ntu.edu.sg)

947

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant growth

Arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] plants were maintained in growth chamber at 22°C under

long-day condition (16 h light/8 h dark). For *Arabidopsis* plants used in ROS assay and Western blot,

plants were kept at short-day condition (8 h light/16 h dark) to facilitate vegetative growth. Col-0, RabF2b-

YFP (Wave2Y), Got1p homolog-YFP (Wave 22Y) seeds were obtained from the Arabidopsis Resource

Center (ABRC) stock centre (Ohio, U.S.A.). *Arabidopsis* FLS2-GFP, pREM1.2::YFP:REM1.2, CLC-GFP,

BRI1-GFP and BOR1-GFP marker lines were described previously (18, 57; 108, 109,110).

Sterol mutants *fkX224*, *smt1-1* and their WT ecotypes were described previously (111, 112). For cell

biology imaging, *Arabidopsis* seeds were surface sterilised with 10% bleach and 70% EtOH, washed

three times with sterilised water and vernalized at 4°C for 2 days. Seedlings were then sown on ½ MS

agar and grown at 22°C for 4-5 days under long-day condition (16 h light/8 h dark) before treatments and

imaging. For growth assay to evaluate primary root growth, we germinated surface-sterilised seeds

directly on ½ MS agar containing indicated drugs. Plants were grown vertically at 22°C for seven days

before images of the plates were taken using a flatbed scanner and primary root length was determined

by Fiji software. Unless specified, all growth assays were repeated at least twice with approximately 20-

30 seedlings for each treatment. For growth assay of sterol biosynthesis mutants on DMSO- and DSF-

supplemented medium, growth inhibition rate (%) were defined as the percent difference between the root

length of DSF-treated plants and the Mean root length of DMSO-treated plants.

Bacterial strains

Pseudomonas syringae pv. *tomato* DC3000 (*Pst*) (113) were cultured at 28 °C on NYG medium.

969

METHODS

971

Chemicals and treatment conditions

DSF (*cis*-11-methyl-2-dodecenonic) was purchased from Merck and dissolved in DMSO to obtain 100 mM

stock. The bacterial flagellin peptides flg22 and flgII-28 were chemically synthesised (GL Biochem Ltd.,

China) with 95% purity and dissolved in water to obtain 5 mM stocks. For pharmacological studies,

Brefeldin A (BFA), Wortmannin, Lovastatin, and ES9-17 were dissolved in DMSO as 50 mM stocks.

Unless specified, seedlings were incubated with these drugs at the working concentration of 50 μM in ½

MS medium for 1 h at room temperature. The sterol-depleting reagent MβCD was dissolved in deionised

979   water at 200 mM and filter-sterilized. For short-term MβCD treatment, *Arabidopsis* seedlings were treated

980   with 2 mM MβCD in liquid ½ MS for 30 min at room temperature. Long-term growth assay with MβCD was

981   performed by germinating axenic *Arabidopsis* seeds directly on ½ MS agar containing 2mM MβCD.

982

983   ***qRT-PCR***

984   *Arabidopsis* Col-0 seeds were surface-sterilized and vernalized as mentioned above. Five-day old

985   seedlings were transferred to new ½ MS plates containing either DMSO or 25 μM DSF for three

986   additional days. At day 8, the seedlings were subjected to a brief treatment with either 150 nM of flg22 or

987   water control and plant tissue was frozen in liquid nitrogen and kept at -80°C. RNA extraction was

988   performed using RNeasy Plant Minikit (Qiagen) according to manufacturer's instruction. Each treatment

989   includes 2-3 biological replicates.

990   DNase treatment was performed twice using Turbo DNase (Ambion) and RNAClean XP kit

991   (Agencourt). The quality of RNA samples was evaluated using the Qubit Fluorometer (Thermo Fisher).

992   cDNA was synthesized by SuperScript III First-Strand Synthesis system (Invitrogen), using oligo-d(T)

993   primer. Quantitative RT-PCR was performed in triplicate for each of the biological replicates, using Kapa

994   SYBR FAST qPCR Master mix with primers specific for *FRK1* (13), and *EF-1α* gene used as an internal

995   control (114). Data were collected by Applied Biosystem StepOnePlus RealTime PCR system (Applied

996   Biosystem) and analyzed using the manufacturer's software.

997

998   **Microscopy and data analysis**

999   *Confocal microscopy*

1000   Confocal microscopy was performed on a Zeiss ELYRA PS.1 + LSM780 system equipped with a

1001   63X Plan-Apochromat oil-objective (NA=1.4). GFP/YFP and FM4-64 were excited with a 488-nm and

1002   514-nm laser, respectively. Emission was collected at 493-594 nm for GFP/YFP and 612-758 nm for

1003   FM4-64, respectively. In addition, chloroplasts autofluorescence in FLS2-GFP cotyledons was also

1004   captured from 630-730 nm.

1005   For imaging of FLS2-containing endosomes, image acquisition was performed as described

1006   previously (115). Drug treatments of FLS2-GFP seedlings were performed in liquid ½ MS as followed:

1007   DSF: 25 μM- 24 h; Sterol mix (Sitosterol:Stigmasterol:Campesterol = 8:1:1): 50 μM- 24 h; MβCD: 2 mM -

1008   30 min (alone or in combination with 25 μM DSF- 24 h); 10 mM – 24 h (alone or in combination with 50

1009 μM sterol mix, 24 h). After drug treatments, FLS2-GFP seedlings were further elicited with 10 μM flg22

1010   and imaged at 30', 60' 120' post-elicitation (water was used as mock control). We also included at

1011   treatment of 10 μM flgII-28 as negative control since flgII-28 peptide is not recognized by *Arabidopsis*

1012   FLS2 receptor. At indicated timepoints, z-stack images of FLS2-GFP seedling cotyledons were taken at

1013   12 slices of 1 μm z-distance. Endosome signals from maximum projection images were segmented to

1014   remove plasma membrane signal using Fiji "Trainable Weka Segmentation" plug-in. Segmented images

1015   were analyzed further by "Particle Analysis" tool in Fiji (116) to obtain the number of endosomes per

1016   image. Experiments were repeated twice, with 12 images taken from three seedlings for each replicate.

1017   To monitor FM4-64 uptakes in PIN2-GFP roots, seeds were sown on ½ MS agar and grown

1018   vertically. Five-day old PIN2-GFP seedlings were then transferred to a new plate containing either DMSO

1019   (control), 25 μM DSF or 50 μM sterol mix (Sitosterol:Stigmasterol:Campesterol = 8:1:1) for an additional

1020   day. After 24 h of growth on DMSO/DSF/Sterol-supplemented media, the following drug treatments were

1021   performed in liquid ½ MS at room temperature: MβCD: 2 mM - 30 min (alone or in combination with 25

1022   μM DSF- 24 h treatment); MβCD: 10 mM – 24 h (alone or in combination with 50 μM sterol mix-24 h

1023   treatment); NaCl: 100 mM - 1h; Mannitol 100mM - 1h; ES9-17: 50 μM-1h; BFA: 50 μM-1h. After

1024   treatments, PIN2-GFP seedlings were incubated with 2 μM FM4-64 for 5 min at room temperature,

1025   washed twice with fresh ½ MS medium and roots were imaged immediately using an ELYRA PS.1 +

1026   LSM780 system equipped with a 63X Plan-Apochromat oil-objective (NA=1.4).

1027   *VA-TIRFM and lifetime analysis*

1028   Variable-angled Total Internal Reflection Fluorescence Microscopy was performed on a Zeiss

1029   ELYRA PS.1 + LSM780 system equipped with a 100X Plan-Apochromat oil-objective (NA=1.46) to

1030   monitor the dynamics of PM markers. Time-lapse images of FLS2-GFP foci on plasma membrane was

1031   taken at up to 120 frames with 1000-ms exposure at 2% laser power. Time-lapse movies of other PM

1032   marker lines were acquired as followed: CLC-GFP: 90 cycles, 1000-ms exposure, 1000-ms interval,

1033   BRI1-GFP and BOR1-GFP: 90 cycles, 500-ms exposure, 500-ms interval. Acquired VA-TIRFM images

1034   were deconvolved using Huygens Essential (Scientific Volume Imaging) using theoretical PSFs. Particle

1035   lifetime was measured from kymographs in Fiji. Lifetime of at least 100 individual foci from three different

1036   seedlings were presented. The clustering of FLS2-GFP foci on plant plasma membrane, indicated by

1037   spatial clustering index (SCI) was measured as described previously (117). Briefly, SCI was calculated

1038   based on the ratio of top 5% pixels with the highest intensity and 5% of pixels with the lowest intensity

1039   along 10-μM line ROIs selected from the first frame of each VA-TIRFM images. Data presented in

1040   boxplots were from at least 60 individual ROIs.

1041

1042   *2D-Structure Illumination Microscopy*

1043   2D-SIM (Structured Illumination Microscopy) image for pREM1.2::YFP:REM1.2 signals was conducted

1044   using a Zeiss ELYRA PS.1 + LSM780 system with a Zeiss Alpha Plan Apochromat 100X (NA=1.46) oil

1045   objective. A 488 nm laser light source was used to excite the YFP signal and the emission was collected

1046   in the range of 495-575 nm by a sCMOS camera (PCO) with a pixel size as 6.5 μm x 6.5 μm, the

1047   exposure time was set as 300 ms. All the 2D-SIM images contained five rotations and five phases of the

1048   grated pattern (42 μm) for each image layer. SIM image reconstruction was conducted by setting the

1049   Super-Resolution (SR) frequency weighting as 0.5 and the noise filter as -4.

1050   Particle tracking was performed in Imaris (Bitplane). Imaris Spots function was used to automatically

1051   quantify the REM1.2 particle signals. To trace all the spots, the estimated spot diameter was set to 0.22

1052 µm according to the preliminary particle analysis before processing the whole image, the threshold was

1053 set to automatic region threshold. After tracing all the particles in each image, the total intensity of each

1054 particle were analyzed and output automatically. For each treatment, data of 500 particles were randomly

1055 selected from more than 10 images (more than 2000 particles in total for each treatment) for further

1056 statistical analysis.

1057

1058 *FLS2-GFP steady-state anisotropy measurement*

1059 Steady state anisotropy were measured on FLS2-GFP expressed live cells in cytoledon using

1060 NikonTE2000 TIRF microscope with dual camera as described previously (38). Briefly, with respect to

1061 plane of polarization of 488 nm excitation light (~ 5.2 mW power), emission intensity was collected by two

1062 Evolve™ 512 EMCCD camera at parallel (I_{pa}) and perpendicular (I_{pe}) direction. Plasma membrane of the

1063 plant was illuminated at HILO using variable TIRF angle module until the best SNR was achieved using a

1064 100X (NA=1.45) objective with image plane pixel size of 106 nm and 1 s exposure time. Anisotropy

1065 measured at HILO (1910, 1950 angles) showed same results as definitive TIRF, using GFP solution and

1066 stable transfected GFP-GPI construct in CHO cells (Fig. S2D-E). Anisotropy values measure at HILO

1067 (2000 angle) even showed similar results if ROI were taken from evenly illuminated area of the cells.

1068 However, we have restricted our all measurements at HILO angle <1950 to nullify any bias due to uneven

1069 illumination. G-Factor of the instrument was calculated with freely diffused FITC solution by calculating

1070 ratio of I_{pa} vs I_{pe} emission intensities (Ghosh et al., 2012). Then anisotropy of the FLS2-GFP was

1071 calculated by G-factor corrected perpendicular images using the following equation:

1072

1073 $$r \text{ (anisotropy)} = \frac{I_{pa}-I_{pe}}{I_{pa}+2I_{pe}}$$

1074 Alignment of images of I_{pa} and I_{pe} and G-factor corrections were done using algorithm written in Matlab

1075 (MathWorks). FLS2-GFP anisotropy in different treatment conditions was compared using data from more

1076 than 150 ROIs (20x20 pixel) from at least eight images taken from at least three seedlings. The

1077 experiment involved two biological replicates.

1078

1079 **Transmission electron microscopy**

1080 *Arabidopsis* Col-0 seeds were surface-sterilized and vernalized at 4°C for two days, then grown

1081 on ½ MS plates at 22°C (16 h light/8 h dark). After five days, the seedlings were transferred to a new ½

1082 MS plates containing either 25 µM DSF or DMSO (solvent control) for an additional three days.

1083 Eight-day-old seedlings were severed into root, hypocotyl and cotyledon sections. Plant tissues were

1084 fixed in primary fixative (1 ml of 2.5% Glutaraldehyde prepared in 25 mM Sodium Cacodylate buffer pH

1085 7.2) overnight at 4°C. Plant tissues were then washed four times in 25 mM sodium cacodylate buffer, 10

1086 min each time. Then, plant tissues were submerged in 1 ml of secondary fixative (2% Osmium tetraoxide,

1087 0.5% Potassium ferricyanide in water) for 2 h at room temperature.

1088   The samples were washed four times with 1 ml of deionized water, each time for 10 min, then
1089   transferred to 2% uranyl acetate solution and incubated at room temperature for 4 h. Plant samples were
1090   washed four times with deionized water, each time for 10 min until the solution became clear. Sample
1091   dehydration was done at room temperature with a series of increasing acetone solution as followed: 30%
1092   - 20 min; 50% - 20 min; 75% - 20 min; 100% - 20 min (2X).

1093   Tissue embedding was performed using Spurr Low-viscosity Embedding Kit (Sigma-Aldrich).
1094   Samples were incubated in increasing Spurr solutions, each time for 1 h as followed: 25%, 50%, 75% and
1095   100%. After incubating with 100% Spurr, the samples were kept in fresh 100% Spurr solution overnight at
1096   4°C. The next day, fresh 100% Spurr was added to the plant samples one more time. Plant tissues were
1097   then transferred into BEEM embedding capsules (Electron Microscopy Sciences) with the position of the
1098   samples at the bottom of the capsules, then incubated for 4 h at RT, followed by another 2 days at 60°C.
1099   Sectioning was performed using a Cryo Leica EM UC7 diatome (Leica, Germany) with sample thickness
1100   set to 70 nm.

1101   Post-staining ultrathin sections were done by incubating the sections with uranyl acetate (in
1102   methanol) for 1 min, then washed with double deionised water. The sections were then incubated with
1103   Reynold's lead citrate solution for 1 min and subsequently washed with deionised water, then dried in a
1104   fume hood. The plant tissue sections were imaged using a Hitachi Transmission Electron Microscope
1105   HT7700 (Hitachi, Japan) with the acceleration voltage of 80 kV.

1106

1107   **Lipidomic profiling**

1108   *Lipid extraction*

1109   Surface-sterilized Col-0 seeds were directly germinated and grown vertically on ½ MS agar
1110   supplemented with DMSO, 25 μM DSF, 2 mM MβCD or DSF+MβCD for 7 days at 22°C, 16 h light/ 8 h
1111   dark. After one week, 30 seedlings were pooled together as one biological replicate and total lipid was
1112   extracted as previously described (118). Prior to lipid extraction, 500 pmol each of PC 28:0, PE 28:0, PS
1113   28:0, PG 28:0, PA 28:0, LPC 20:0, LPE 14:0, LPA 17:0, SM 12:0, Cer 17:0, CerOH 12:0, HexCer 8:0, TG
1114   48:0 d5, GB3 23:0, and cholesterol d6 were added to each sample as internal standards. The experiment
1115   involved six biological replicates for each treatment.

1116

1117   *LC-MS analysis*

1118   Dried lipid extracts were resuspended in 500 μL Butanol-Methanol (1+1, v/v) and stored at -20 °C
1119   until LC-MS analysis. Phospholipids, sphingolipids, and sterol lipids were identified using an Agilent 1290
1120   UHPLC system coupled to an Agilent 6495 triple quadrupole mass spectrometer. Chromatographic
1121   separation was performed on an Agilent Eclipse Plus C18 reversed phase column (50 x 2.1mm, particle
1122   size 1.8 μm) using gradient elution of solvent A (water/acetonitrile 60/40, v/v, 10 mM ammonium formate)
1123   and solvent B (isopropanol/acetonitrile 90/10, v/v, 10 mM ammonium formate). 20% solvent B was

1124 increased to 60 % B over 2 min, and to 100 % B over 5 minutes. The column was flushed with 100 % B

1125 for 2 minutes, and then reequilibrated with 20 % B for a further 1.8 minutes. The flow rate was 0.4

1126 mL/min, column temperature was 50 °C, and injection volume was 1 μL. The JetStream ion source was

1127 operated at the following conditions: Gas temperature, 200 °C; gas flow, 12 L/min; nebulizer gas

1128 pressure, 25 psi; sheath gas heater temperature, 400 °C; sheath gas flow, 12 arbitrary units; capillary

1129 voltage, 3500 V for positive ion mode, and 3000 V for negative ion mode; Vcharging, 500 for positive ion

1130 mode, and 1500 for negative ion mode; positive high pressure RF, 200; negative high pressure RF, 90;

1131 positive low pressure RF, 100; negative low pressure RF, 60; fragmentor voltage, 380 V; cell accelerator

1132 voltage, 5 V; dwell time, 1 ms.

1133 The injection order of 24 samples (six replicates of four treatment groups) was randomised, and

1134 bracketed by injections of pooled quality control and extracted blank samples to monitor system stability

1135 and carryover. The whole sample set was analysed twice consecutively with different SRM settings. One

1136 injection was for phospholipids and sterols using SRM transitions from Vu *et al.* (118), and the second

1137 method detected plant sphingolipids with SRM transitions adapted from Markham et al. (119).

1138 *Data processing*

1139 Lipids were analysed based on characteristic SRM transitions and retention time using Agilent

1140 MassHunter Quantitative Analysis for QQQ (v. B.07.00). Further processing, including normalisation to

1141 suitable internal standards was performed in Microsoft Excel. High-resolution LC-MS data was processed

1142 using XCalibur QuanBrowser 3.0.63 (Thermo Fisher).

1143

1144 **Immunoblot analysis**

1145 Three leaves discs (0.7 cm in diameter) from 5 to 6-week old *Arabidopsis* plants were cut into

1146 strips and floated for 24 h in sterile water in 24-well plates under constant light to reduce stress due to

1147 wounding. DMSO or DSF were added into the wells 24 h before elicitation with 10 μM flg22. Leaf tissue

1148 was washed with sterile water to remove the elicitor and flash-frozen in liquid nitrogen at different

1149 timepoints. Total protein extraction was performed as described previously (85).

1150 To detect the flagellin receptor, we used α-GFP antibody (1:200; Torrey Pines BioLabs, NJ,

1151 U.S.A) to probe for the FLS2-GFP in FLS2-GFP transgenic plants. For monitoring p-MAPK response, we

1152 used α-p-MAPK antibody (1:3000; Cell Signalling Technology, MA, U.S.A) to detect phosphorylated

1153 MAPKs in Col-0 leaf tissue after flg22 peptide elicitation. Western blot membranes were stained with

1154 Ponceau S as internal loading control.

1155

1156 **Measurement of callose deposition**

1157 Measurement of plant callose deposition in response to bacterial flagellin peptide was performed

1158 on two-week old Col-0 seedlings germinated on ½ MS medium. Whole seedlings were individually placed

1159 in 6-well plates with 4 ml of liquid ½ MS supplemented with DMSO or 25 μM DSF for 24 h at 22°C, then

31

1160   elicited for 24 h with 1 μM of flg22. Challenged plants were destained with acetic acid:ethanol (1:3)

1161   overnight, then washed in 150 mM K_2HPO_4 for 30 min. Destained leaves were incubated in 0.01% aniline

1162   blue (dissolved in 150 mM K_2HPO_4) in the dark for 2 h, then mounted on microscope slides in 50%

1163   glycerol. Callose deposition were observed on a Zeiss ELYRA PS.1 + LSM780 system using a DAPI filter

1164   (excitation:emission, 370:509 nm) with a 10X objective. The experiment was repeated twice, each

1165   involved 8 individual plants and at least 6 images were taken from each plant.

1166

1167   **Stomatal closure assay**

1168   Five-week-old Col-0 plants were used to determine the stomatal closure response in response to

1169   the flg22 peptide. Briefly, two-three leaves/plant were marked with a permanent marker, then infiltrated

1170   with DMSO or 25 μM DSF (diluted in sterilised 10 mM $MgCl_2$). The plants were returned to the growth

1171   chamber for an additional 24 h. The next day, infiltrated leaves were excised from the plants and the

1172   abaxial epidermis was peeled off and transferred to a petri disc containing sterile water using a natural-

1173   hair brush (size 8). 10 μM flg22 was added to the samples for 30 min. The epidermal peels were stained

1174   with 20 μM propidium iodine for 5 min and rinsed briefly with sterile water, then mounted on microscope

1175   slides without coverslips and examined under a wide-field fluorescence microscope (Leica) equipped with

1176   RFP filter using a 10X objective lense. The experiments involved measurements of $\geq$130 stomata from at

1177   least 10 images taken from five individual plants.

1178

1179   **ROS production**

1180   Measurement of ROS burst in *Arabidopsis* apoplast elicited by flg22 was performed as described

1181   previously (24). Briefly, each leaf disc (0.7 cm in diameter) from 5 to 6-week old *Arabidopsis* plants was

1182   cut into 5 strips, floated on sterile water for 24 h in white 96-well plates under constant light at room

1183   temperature to reduce stress from wounding. Drugs were added into the wells prior to elicitation with 1

1184 μM flg22 as followed: 25 μM DSF/DMSO control, 24 h; 2 mM MβCD, 30 min; 50 μM sterol mix, 24 h; 1 μM

1185   Lovastatin, 24 h. Each ROS experiment was performed in duplicate and experiments showing on the

1186   same graph were performed in the same plate.

1187

1188   **Plant infection assay**

1189   Two-week old *Arabidopsis* plants grown on ½ MS agar was used for flood-inoculation with

1190   *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Using *Pst* DC3000, a bacterial pathogen with

1191   a different quorum sensing system other than DSF, allowed us to dissect the effect of DSF on plant

1192   immunity independent of effector proteins activation and other bacterial traits regulated by quorum

1193   sensing. Briefly, plants were flooded with 40 ml of 10 mM $MgCl_2$ solution containing 25 μM DSF or DMSO

1194   (control). After 1 day, the liquid was decanted and another 40 ml of $MgCl_2$ containing either 1 μM of flg22

1195   or water was poured into each plate. On the next day, the seedlings were inoculated with 5×10^6 CFU/ml

1196   of *Pst* DC3000 prepared in 10 mM $MgCl_2$. Bacterial population was determined 4 days post-inoculation

1197   (DPI) by dilution plating as described previously (120). The experiment was repeated twice with 9-12

1198   technical replicates/treatment.

1199   **Co-immunoprecipitation**

1200   The constructs for transient expression of BAK1 and FLS2 have been described earlier (93).

1201   Protoplast-based co-IP assays were conducted as previously described (121). Briefly, 1 ml of transfected

1202   protoplasts were incubated for 12 h for protein expression in the presence of 1 μM DSF (Sigma, USA),

1203   and were treated with or without 1 μM flg22 (MoonBiochem, China) for 10 min before harvest. Briefly,

1204   cells were lysed in 500 μl IP buffer [10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol,

1205   1% Triton-X100, and 1× protease inhibitor cocktail (Roche, USA)], and 30 μl of protein lysate was kept as

1206   the input fraction. The remaining 470 μl lysate was incubated with 10 μl anti-FLAG M2 affinity gel (Sigma,

1207   USA) at 4°C for 3 h. After being washed five times with ice-cold IP buffer and once with 50 mM Tris-HCl

1208   (pH 7.5), the bound proteins were solubilized by boiling the beads in 60 μl 2×SDS-PAGE loading buffer,

1209   and were detected by immunoblotting using anti-FLAG (Sigma, USA) or anti-HA antibody (Roche, USA).

1210   The experiment was repeated twice.

1211

1212   **QUANTIFICATION AND STATISTICAL ANALYSIS**

1213   Statistical analyses and data visualisation were performed using GraphPad Prism 7.0 (GraphPad,

1214   CA, U.S.A.). Mean comparisons were performed using one-way ANOVA ([*] $p < 0.05$; [**] $p < 0.01$; [***] $p <$

1215 0.001; [****] $p < 0.0001$; ns = not significant). Unless specified, data were presented as box-and-whiskers

1216   plots with bars indicated the median, 25% and 75% quartiles.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6