The bacterial virulence factors rhamnolipids and their (*R*)-3-hydroxyalkanoate precursors activate *Arabidopsis* innate immunity through two independent mechanisms

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Abstract

Plant innate immunity is activated upon perception of invasion pattern molecules by plant cell-surface immune receptors. Several bacteria of the genera *Pseudomonas* and *Burkholderia* produce rhamnolipids (RLs) from L-rhamnose and (*R*)-3-hydroxyalkanoate precursors (HAAs). RL and HAA secretion is required to modulate bacterial surface motility, biofilm development, and thus successful colonization of hosts. Here, we show that the lipidic secretome from the opportunistic pathogen *Pseudomonas aeruginosa* mostly comprising RLs and HAAs stimulates *Arabidopsis* immunity. We demonstrate that HAAs are sensed by the bulb-type lectin receptor kinase LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION/S-DOMAIN-1-29 (LORE/SD1-29) that also mediates medium-chain 3-hydroxy fatty acid (mc-3-OH-FA) perception in the plant *Arabidopsis thaliana*. HAA sensing induces canonical immune signaling and local resistance to plant pathogenic *Pseudomonas* infection. By contrast, RLs trigger an atypical immune response and resistance to *Pseudomonas* infection independent of LORE. Thus, the glycosyl moieties of RLs, albeit abolishing sensing by LORE, do not impair their ability to trigger plant defense. In addition, our results show that RL-triggered immune response is affected by the sphingolipid composition of the plasma membrane. In conclusion, RLs and their precursors released by bacteria can both be perceived by plants but through distinct mechanisms.

Significance

Activation of plant innate immunity relies on the perception of microorganisms through self and nonself elicitors. Rhamnolipids and their precursor HAAs are exoproducts produced by beneficial and pathogenic bacteria. They are involved in bacterial surface dissemination and biofilm development. As these compounds are released in the extracellular milieu, they have the potential to be perceived by the plant immune system. Our work shows that both compounds independently activate plant immunity. We demonstrate that HAAs are perceived by the receptor protein kinase LORE. By contrast, rhamnolipids are not senses by LORE but activate a non-canonical immune response affected by the sphingolipid composition of the plant plasma membrane. Thus, plants are able to sense bacterial molecules as well as their direct precursors to trigger a distinct immune response.

Introduction

Plant innate immunity activation relies on detection of invasion pattern (IP) molecules that are perceived by plant cells (1, 2). Non-self-recognition IPs include essential components of whole classes of microorganisms, such as flagellin, peptidoglycans, mc-3-OH-FAs from bacteria or chitin and β-glucans from fungi and oomycetes, respectively (3, 4). Apoplastic IPs are sensed by plant plasma membrane-localized receptor kinases (RKs) or receptor-like proteins (RLPs) that function as pattern recognition receptors (PRRs) (5, 6). Activation of the immune response requires the recruitment of regulatory receptor kinases and receptor-like cytoplasmic kinases (RLCKs) by PRRs (7). Early cellular immune signaling of pattern-triggered immunity (PTI) includes ion-flux changes at the plasma membrane, rise in cytosolic Ca^{2+} levels, production of extracellular reactive oxygen species (ROS) and activation of mitogen-activated protein kinases (MAPKs) and/or Ca^{2+}-dependent protein kinases (3, 8-10). Biosynthesis and mobilization of plant hormones, including salicylic acid, jasmonic acid, ethylene, abscisic acid and brassinosteroids, ultimately modulate plant resistance to phytopathogens (11-14).

Rhamnolipids (RLs) are extracellular amphiphilic metabolites produced by several bacteria, especially *Pseudomonas* and *Burkholderia* species (15-17). Acting as wetting agents, RLs are essential for the social form of bacterial surface dissemination called swarming motility and for normal biofilm development (18-20). These glycolipids are produced from L-rhamnose and 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) precursors (15, 21). HAAs are synthesized by dimerization of (*R*)-3-hydroxyalkanoyl-CoA in *Pseudomonas*, forming congeners through the RhlA enzyme (21). The opportunistic plant pathogen *Pseudomonas aeruginosa* and the phytopathogen *Pseudomonas syringae* produce extracellular HAAs (16, 22-24). In *P. syringae*, HAA synthesis is coordinately regulated with the late-stage flagellar gene encoding flagellin (22). HAA and RL production is finely tuned and modulates the behavior of swarming migrating bacterial cells by acting as self-produced negative and positive chemotactic-like stimuli (25). RLs contribute to the alteration of the bacterial outer membrane composition, by shedding flagellin from the flagella (26) and by releasing lipopolysaccharides (LPS) resulting in an increased hydrophobicity of the bacterial cell surface (27). In mammalian cells, RLs produced by *Burkholderia plantarii* exhibit endotoxin-like properties similar to LPS, leading to the production of proinflammatory cytokines in human mononuclear cells (28, 29). They also subvert the host innate immune response through manipulation of the human beta-defensin-2 expression (30). Moreover, RLs from *Burkholderia pseudomallei* induce Interferon gamma (IFN-γ)-dependent host immune response in goat (31).

In plants, RLs induce defense responses and resistance to biotrophic and necrotrophic pathogens (32, 33). They also contribute to the biocontrol activity of the plant beneficial bacterium *P. aeruginosa* PNA1 against oomycetes (17). Recently, it was reported that the bulb-type lectin receptor kinase LIPOOLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION/S-DOMAIN-1-29 (LORE/SD1-29) mediates medium-chain 3-hydroxy fatty acid (mc-3-OH-FA) sensing in *Arabidopsis thaliana* (hereafter, *Arabidopsis*) and that bacterial compounds comprising mc-3-OH-acyl building blocks including LPS and RLs do not stimulate LORE-dependent responses (34).

Here we show that the lipidic secretome produced by *P. aeruginosa* (RLsec) mostly composed of RLs and HAAs induces *Arabidopsis* immunity. HAAs are perceived through the RK LORE. We demonstrate that, albeit not being sensed by LORE, RLs trigger an immune response characterized by an atypical defense signature. Altogether, our results demonstrate that RLs and their precursors produced by *Pseudomonas* bacteria stimulate the plant immune response by two distinct mechanisms.

70

71 ## Results

72 **RLsec from *Pseudomonas* induces *Arabidopsis* immune responses partially mediated by LORE.**
73 *Pseudomonas* species including opportunistic plant pathogenic or plant beneficial endophytic strains release a
74 mixture of RL congeners and HAA precursors, here collectively termed RL secretome (RLsec) (15, 25). HPLC-
75 MS/MS analyses of this RLsec from *P. aeruginosa* revealed the presence of mono-RLs and di-RLs at 50.9%
76 and 44.9% of dry weight, respectively, and HAAs (3.8% of dry weight) (Supplementary Table 1). RLs comprising
77 ten-carbon long lipid tails, Rha-C_{10}-C_{10} (α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate) and
78 Rha-Rha-C_{10}-C_{10} (α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate), and
79 C_{10}-C_{10} [(*R*)-3-(((*R*)-3-hydroxydecanoyl)oxy)decanoate] HAAs were the most abundant molecules in this lipidic
80 secretome (37.6%, 33.1%, 2.1%, respectively). Notably, low amounts of free mc-3-OH-FAs (0.4% total), such
81 as 3-OH-C_8, 3-OH-C_{10} and 3-OH-C_{12}, were also identified (Supplementary Table 1).

82 First, we monitored apoplastic ROS production triggered by RLsec in *Arabidopsis* (35). Wild type (WT)
83 plants challenged with RLsec displayed a transient extracellular ROS production, starting at six minutes and
84 peaking at 15 minutes post elicitation (Fig. 1A). A robust ROS response was detected at concentrations of
85 RLsec starting from 0.5 µg/mL (Fig. 1B, Supplementary Fig. 1). The ROS burst was dependent on the
86 transmembrane- NADPH oxidase RBOHD (36, 37) (Fig. 1C, Supplementary Fig. 2). RKs and RLPs mediate
87 perception of IPs and early activation of PTI signaling (7). We monitored RLsec-triggered ROS production in
88 *Arabidopsis* plants carrying loss-of-function mutations in genes encoding well characterized RKs and RLPs
89 *fls2/efr1* (38, 39), *bak1*-5, *bkk1*-1, *bak1*-5/*bkk1*-1 (40), *bik1/pbl1* (41), *cerk1*-2 (42), *sobir1*-12, *sobir1*-13 (43),
90 *dorn1*-1 (44) and *lore*-5 (45). RLsec-induced production of ROS was only reduced in *lore*-5 (Fig. 1C,
91 Supplementary Fig. 2). Some IPs, including LPS extracts and synthetic mc-3-OH-FAs, were reported to induce
92 a late ROS production in *Arabidopsis* (34, 46, 47). The late ROS response triggered by mc-3-OH-FAs was
93 dependent on LORE (34). RLsec also induced a late and long-lasting ROS burst in *Arabidopsis* culminating at
94 6-8 hours post treatment (Fig. 2A), which was abolished in *rbohD* but not in *lore-5* mutant plants (Fig. 2A).

95 Next, we tested whether RLsec induces local resistance to the hemibiotrophic phytopathogen
96 *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst)* in *Arabidopsis* (48). RLsec pretreatment significantly
97 enhanced resistance against *Pst* infection in WT leaves and, although less pronounced, in *lore*-5 plants (Fig.
98 2B). Taken together, our results show that RLsec induces immunity-related signaling events and disease
99 resistance in *Arabidopsis* that are partially mediated by the bulb-type lectin RK LORE.

100

101 ***Pseudomonas* HAAs and mc-3-OH-FAs from RLsec trigger LORE-dependent *Arabidopsis* immunity.**

102 By contrast to RLsec, purified RLs do not trigger LORE-dependent $[Ca^{2+}]_{cyt}$ and early ROS signaling
103 responses (34). Because RLsec contains significant amounts of HAAs, we investigated the role of these poorly
104 studied compounds in RLsec-triggered immunity. We compared the responses to HAA with those to mc-3-OH-
105 FAs, known to be sensed by LORE (34) and present in low amounts in RLsec (Supplementary table 1). Side-
106 by-side experiments with C_{10}-C_{10} HAA purified from *Pseudomonas aeruginosa* secretome and 3-OH-C_{10}
107 revealed that both compounds induce $[Ca^{2+}]_{cyt}$ signaling and ROS production in WT plants in a dose-dependent
108 manner (Fig. 3A and 3B, Supplementary Fig. 3 and 4). As observed upon 3-OH-C_{10} elicitation, purified C_{10}-C_{10}-
109 induced ROS response was impaired in *rbohD* and *lore*-5 mutants (Fig. 3C). Similarly, $[Ca^{2+}]_{cyt}$ signaling

110 triggered by C_{10}-C_{10} was impaired in *lore*-5 (Fig. 3D). In addition, C_{10}-C_{10} and 3-OH-C_{10} both triggered LORE-
111 dependent MPK3 and MPK6 phosphorylation (Supplemental Fig. 5A). C_{10}-C_{10} activated a late and long-lasting
112 ROS production which, unlike the RL-triggered ROS burst, was LORE-dependent (Supplemental Fig. 6). WT
113 but not *lore*-5 mutant plants pretreated with C_{10}-C_{10} or 3-OH-C_{10} displayed enhanced resistance against *Pst*
114 (Fig. 3E). Similar to 3-OH-FAs (34), the acyl chain length of HAA affects its immune eliciting activity, as purified
115 C_{14}-C_{14} from *B. glumae* did not induce ROS production in *Arabidopsis* plants (Supplementary Fig. 7).
116 Trace amount of 3-OH-C_{10} was detected in C_{10}-C_{10} purified from *P. aeruginosa* RLsec (Supplementary
117 Table 2). To avoid any influence of potential contamination of HAAs with eliciting compounds related to
118 purification procedure, we tested chemically synthesized C_{10}-C_{10} for the ROS and $[Ca^{2+}]_{cyt}$ responses. Synthetic
119 C_{10}-C_{10} triggered LORE-dependent $[Ca^{2+}]_{cyt}$ signaling and ROS production in a dose-dependent manner (Fig.
120 4A-C). WT plants pretreated with synthetic C_{10}-C_{10} also displayed LORE-dependent enhanced resistance
121 against *Pst* infection (Fig. 4D).
122 Altogether, our results show that HAAs secreted by *Pseudomonas* are sensed by *Arabidopsis* through
123 the bulb-type lectin RK LORE, activate canonical PTI-related immune responses and provide resistance to
124 bacterial infection.
125
126 **RLs trigger LORE-independent *Arabidopsis* immune responses and resistance to *Pst*.**
127 To investigate whether RLs activate a LORE-independent immune response, we used purified Rha-
128 Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10}, the most abundant molecules from *P. aeruginosa* RLsec. In *Arabidopsis* WT,
129 both RL congeners induced a late and long-lasting ROS production, but as observed previously (34), no early
130 burst (Fig. 5A). As both RL congeners gave a similar ROS signature, we only used Rha-Rha-C_{10}-C_{10} in the
131 following experiments. The minimal concentration necessary to stimulate ROS production was 50 µM with an
132 optimum at 100 µM (Fig. 5B). Late ROS production was compromised in *rbohD* but not in *lore*-5 mutants (Fig.
133 5C). Surprisingly, neither MPK3 nor MPK6 activation by Rha-Rha-C_{10}-C_{10} was detectable over a 3-hour time-
134 course (Supplementary Fig. 5B). L-Rhamnose alone was inactive demonstrating that the lipid part of the RLs is
135 necessary to trigger the immune response (Fig. 5A). *Burkholderia* species produce RL congeners with longer
136 lipid chains than those produced by *Pseudomonas* (15). The RLsec from phytopathogenic *Burkholderia glumae*
137 only contains congeners with fatty acid chain lengths varying from 12 to 16 carbons, in particular Rha-Rha-C_{14}-
138 C_{14} (49, 50). Challenge of *Arabidopsis* with purified Rha-Rha-C_{14}-C_{14} from *B. glumae* did not trigger any ROS
139 production (Fig. 5A) suggesting that the length of the fatty acid chain of RLs is critical for their eliciting activity.
140 To determine whether RLs trigger local resistance to pathogenic *Pseudomonas* independent of LORE, plants
141 were pretreated with 10 µM purified Rha-Rha-C_{10}-C_{10} before *Pst* inoculation. WT plants displayed a significant
142 enhanced resistance against *Pst* that was not compromised in *lore*-5 mutants (Fig. 5D).
143 To get deeper insights into the mechanisms involved in RL sensing, we used *Arabidopsis* plants carrying
144 loss-of-function mutations in genes encoding RK and RLPs but also plasma membrane channel mutants
145 including quintuple mechano-sensitive channels of small conductance-like (*msl4/5/6/9/10*) and double mid1-
146 complementing activity (*mca1/2*) channel mutants (51) that could monitor changes in membrane mechanical
147 properties. None of these mutants were affected in the long-term ROS response (Fig. 6A). Glycosylinositol
148 phosphorylceramide (GIPC) sphingolipids were recently involved in the sensing of microbial necrosis and
149 ethylene-inducing peptide 1-like (NLP) proteins (52). We found that the fatty acid hydroxylase *fah1/2* mutant

150 that is disturbed in its complex sphingolipid composition (52) showed a reduced long-term ROS response (Fig.
151 6B). Ion leakage measurement confirmed that *fah1/2* mutant plants were less affected than WT plants by RL
152 treatment (Fig. 6C). Ceramide synthase *loh1* mutants are also impaired in GIPC levels but not in glucosyl
153 ceramides (52). Interestingly, RL-triggered ROS production and ion leakage was unaltered in *loh1* plants.
154 Altogether, our results show that RLs activate an atypical immune response in *Arabidopsis* that is LORE-
155 independent, but which is affected by the sphingolipid composition of the plasma membrane.
156

157 **Discussion**

158 In *Pseudomonas* and *Burkholderia* species, swarming motility is intimately related to the production of
159 extracellular surface-active RLs and HAAs (22, 25, 53-55). In addition, RL production affects bacterial biofilm
160 architecture and increases affinity of cells for initial adherence to surfaces through increasing the cell's surface
161 hydrophobicity (19, 56). These exoproducts are therefore at the frontline during host colonization. Our work
162 demonstrates that both RLs and HAAs from the *Pseudomonas* lipidic secretome, referred to as RLsec here, are
163 able to trigger *Arabidopsis* innate immunity by two distinct mechanisms.
164 We found that *Pseudomonas* RLs induce an atypical immune response. This response does not involve
165 the RK LORE. Other bacterial compounds comprising mc-3-OH-acyl building blocks, but with large decorations
166 including lipid A or LPS, lipopeptides, and *N*-acyl homoserine lactones also do not trigger LORE-dependent
167 immune responses (34). RLs are glycolipids made of L-rhamnose linked to an HAA lipid tail (15, 21). Therefore,
168 glycosylation of HAAs abolishes their perception by LORE. Glycosylation is known to affect the perception of
169 IPs. Glycosylation of flg22 from *Acidovorax avenae* on Ser^{178} and Ser^{183} prevents its perception by rice cell (57).
170 Similarly, unglycosylated flagellin from *Pseudomonas syringae* pv. *tabaci* 6605 induces stronger defense
171 responses in tobacco plants than glycosylated flagellin (58). In humans, glycosylation of *Burkholderia*
172 *cenocepacia* flagellin significantly reduces its perception by epithelial cells (59).
173 We found that RL perception does not involve previously characterized RKs, RLPs or mechanosensitive
174 channels. However, the RL response is affected by alterations in sphingolipid synthesis suggesting a role of
175 these key membrane lipids in RL-triggered immunity. Recently GIPCs, major structural components of the plant
176 plasma membrane together with glucosylceramides (GlcCers), have been involved as receptors of cytotoxic
177 NLPs (52). NLPs bind terminal monomeric hexose moieties of GIPCs. Only eudicot plants are sensing these
178 NLPs through sphingolipid receptors. Insensitivity of monocots to NLPs is due to the length of the GIPC
179 headgroup, consisting of three terminal hexoses compared to two in eudicots (52). *fah1/2* mutants display
180 reduced glycosylsphingolipids (GIPCs and GlcCers) content but also lower level of ordered plasma membranes
181 (52), suggesting that, similar to the NLP response, complex sphingolipids and/or ordered plasma membranes
182 are necessary for the RL response. Unlike NLPs, RL responses were not significantly affected in *loh1* mutant
183 plants also suggesting that GlcCers more than GIPC could influence RL sensing (52). Surfactin and more
184 recently synthetic RL bolaforms and synthetic glycolipids, also active in the micromolar range, have been
185 proposed to directly interact with plasma membrane lipids (46, 60-62). Mono- and di-RLs from *Pseudomonas*
186 interact with phospholipids in several model membranes (63-66). In particular, RLs are able to fit into
187 phospholipid bilayers of plant membrane model (67). In this model, the rhamnose polar heads from RLs are
188 located near the phosphate groups from phospholipids and RL hydrophobic lipid tails are surrounded by the
189 lipid chains from these phospholipids (67). The results obtained with these plant plasma membrane models

190 suggest that the insertion of RLs into the lipid bilayer does not significantly affect lipid dynamics. The nature of
191 the phytosterols could however influence the RL effect on plant plasma membrane destabilization. Subtle
192 changes in lipid dynamics could then be linked to plant defense induction (67). Interestingly, RL bolaforms, like
193 natural RLs are inducing a non-canonical defense signature with a long-lasting oxidative burst without MPK3 or
194 MPK6 activation (46). This atypical defense signature triggered by two structurally different RLs, displaying
195 amphiphilic properties and biological activities at the micromolar range, could suggest a direct interaction of
196 these molecules with plant plasma membrane lipids.

197 We also demonstrated that HAAs, found in large amount in *Pseudomonas* lipidic secretome, are IPs
198 perceived by *Arabidopsis*. HAA sensing is mediated by LORE (34). HAAs, in the micromolar range, induce
199 typical PTI responses including transient ROS production, $[Ca^{2+}]_{cyt}$ signaling, and MPK3 and MPK6
200 phosphorylation in *Arabidopsis.* Interestingly, 3-OH-C_{10} activates similar responses but at concentrations 10 to
201 50 times lower. This is intriguing, because HAAs are present in much larger quantities (more than 3%) compared
202 to 3-OH-FAs (0.3%) in the lipid secretome (Supplemental table 1). This high amount of HAAs could therefore
203 compensate for their lower activity. RLs are activating an immune response at relatively high concentrations
204 compared to both compounds. Interestingly, the RL concentration in the *P. aeruginosa* lipidic secretome is 10
205 to 100 times higher than HAAs and usually in the millimolar range (23, 68). RLs are produced between 20 and
206 110 µM *in vivo* in mammals infected by *P. aeruginosa*, especially during cystic fibrosis disease (69-71). The
207 high concentrations of RLs needed for plant elicitation are in the range of the concentrations produced by the
208 bacteria.

209 Higher steric hindrance of HAA compared to 3-OH-FAs likely results in a lower affinity to the LORE
210 receptor. Synthetic ethyl 3-hydroxydecanoate (Et-3-OH-C10:0) and *n*-butyl 3-hydroxydecanoate (*n*But-3-OH-
211 C10:0), which possess unbranched ester-bound carbon chains in place of the carboxyl group, also triggered
212 LORE-dependent immune signaling in *Arabidopsis*, while 3-branched *tert*-butyl 3-hydroxydecanoate (*t*But-3-
213 OH-C10:0) was inactive (34). HAAs, possessing a 2-branched ester-bound headgroup, activate LORE signaling.
214 The differences in efficacy could be explained by the different steric hindrance of the molecules. Alternatively,
215 the additional carboxyl group could account for the LORE-eliciting activity of HAAs.

216 *Pantoea*, *Dickeya* and *Pseudomonas* bacteria, in particular the well-known phytopathogen *P. syringae*
217 mainly produce HAAs containing 3-hydroxydecanoic acid (C_{10}) tails (15, 22, 72). By contrast, *Burkholderia*
218 species including the phytopathogenic bacterium *B. glumae*, mainly produce HAAs comprising 3-
219 hydroxytetradecanoic acid (C_{14}) tails (49). *Pseudomonas* C_{10}-containing HAAs activated *Arabidopsis* PTI
220 whereas *Burkholderia* HAAs containing C_{14} fatty acid did not. Chain-length specificity was also observed for
221 mc-3-OH-FA sensing by the LORE receptor with 3-OH-C_{10} representing the strongest immune elicitor (34). Thus,
222 it could be hypothesized that *Arabidopsis*, and more generally *Brassicaceae* (73), are able to specifically
223 recognize HAAs from specific bacterial species, among which several are plant opportunistic and
224 phytopathogens (74-77). Interestingly, transcript profiles of the bean pathogen *P. syringae* pv. *syringae* B728a
225 support a model in which leaf surface or epiphytic sites specifically favor swarming motility based on HAA
226 surfactant production (55, 78). Low levels of HAAs contributing to motility are produced by these bacteria (22).
227 HAA concentrations necessary to stimulate *Arabidopsis* innate immunity are in line with the concentration
228 detected in RLsec and are produced by *Pseudomonas* (between 3 to 20% of the secretome) (23, 68, 79).

229 Low amounts of free mc-3-OH-FAs were found in RLsec from *P. aeruginosa* (Supplementary table 1).
230 In *Pseudomonas*, the outer membrane lipase PagL releases 3-OH-C_{10} during synthesis of penta-acylated lipid
231 A (34). The further fate of this 3-OH-C_{10} is unknown. RLs are able to extract LPS from the outer membrane of
232 *P. aeruginosa* (27). Conceivably, surface-active RLs, and presumably also HAAs, could release free 3-OH-C_{10},
233 produced through PagL activity, along with LPS from the bacterial cell wall or outer membrane vesicles (27).
234 Alternatively, degradation of HAAs/RLs *in planta* may also release free 3-OH-C_{10}. Acyl carrier protein (ACP)-
235 and coenzyme A (CoA)-bound mc-3-OH-FAs are precursors of HAA/RL synthesis (21). Upon bacterial cell lysis,
236 enzymatic or non-enzymatic degradation processes may also generate free 3-OH-C_{10} from these precursors.
237 *In vivo*, insights into IP release have been recently obtained for flagellin. The plant glycosidase BGAL1 facilitates
238 the release of immunogenic peptides from glycosylated flagellin, upstream of cleavage by proteases (80). The
239 pathogen may evade detection by altering flagellin glycosylation and inhibiting the plant glycosidase. Flagellin
240 glycosylation increases its physical stability that could contribute to the non-liberation/recognition of the flg22
241 epitope (58, 81). RLs are able to shed flagellin from *P. aeruginosa* flagella (26), suggesting that these
242 biosurfactants participate in the release of this and presumably other eliciting compounds.
243 In conclusion, we hypothesize that when HAA- and RL-producing *Pseudomonas* colonize the leaf or
244 root surface, they release RLs and HAAs which are necessary for surface motility, biofilm development, and
245 thus successful colonization. Whereas *Arabidopsis* senses HAAs and mc-3-OH-FAs through the bulb-type lectin
246 receptor kinase LORE, RLs are perceived through a LORE-independent mechanism. In addition to direct
247 activation of a non-canonical defense response in plants, RLs, by releasing other IPs from bacteria, could
248 orchestrate a node leading to strong activation of plant immunity.
249
250
251 ## Methods
252 **Molecules.** The *P. aeruginosa* lipidic secretome used in this study was obtained from Jeneil Biosurfactant Co.,
253 Saukville, USA (JBR-599, lot. #050629). Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{10} were purified from this lipidic
254 secretome mixture, as previously described (33, 34). Rha-Rha-C_{14}-C_{14} were purified from the *B. glumae* lipidic
255 secretome (49). To obtain pure HAAs from *P. aeruginosa* or *B. glumae*, RLs were hydrolyzed using 1 M HCl in
256 1:1 dioxane-water boiling at reflux for 60 min. The mixture was extracted with ethyl acetate and the extracts
257 were dried over anhydrous Na_2SO_4. After filtration, the resulting extracts were then evaporated to dryness and
258 resuspended in 2 mL of methanol. HAAs were then isolated from digested mixture using flash chromatography
259 on a Biotage (Stockholm, Sweden) Isorela One instrument with a SNAP Ultra C18 12g column (Biotage) using
260 an acetonitrile/water gradient at 12 mL/min flow rate. The elution was started with 0% acetonitrile for 4.5 min
261 and the acetonitrile concentration was raised to 100% over 28.2 min, followed by an isocratic elution of 100%
262 acetonitrile for 13.3 min. The flash chromatography fraction containing the C_{10}-C_{10} was further separated and
263 purified using 0.25 mm thin-layer chromatographic (TLC) plates (SiliCycle SilicaPlate F-254) and developed
264 with *n*-hexane-ethyl acetate-acetic acid (24:74:2). The bands were scraped from the plates and the HAAs,
265 including C_{10}-C_{10}, were extracted from the silica with chloroform-methanol (5:1). 3-OH-C_{10} was purchased from
266 Sigma-Aldrich Saint-Quentin Fallavier, France. All compounds were dissolved in ethanol or methanol as
267 indicated to prepare stock solutions. Final aqueous compound dilutions were prepared freshly on the days of
268 the experiment. Control solutions contained equal amounts of ethanol or methanol (0.05% for most experiments

269 and not exceeding 0.5% for the highest concentrations tested). Chemical synthesis of C_{10}-C_{10} is described in
270 supplementary data 1 and 2.
271
272 **LC-MS analysis of HAAs.** Samples were prepared by diluting stock solutions using MeOH to final concentration
273 of 50 ppm. 16-Hydroxyhexadecanoic acid at 20 ppm was added to samples as internal standard[71]. The analyses
274 were performed with a Quattro II triple quadrupole mass spectrometer (Micromass, Pointe-Claire, Canada)
275 equipped with a Z-spray interface using electrospray ionization in negative mode. The capillary voltage was set
276 at 3.5 kV and the cone voltage at 25 V. The source temperature was kept at 120°C and the desolvation gas at
277 150°C. The scanning mass range was from 130 to 930 Da. The instrument was interfaced to a high-performance
278 liquid chromatograph (HPLC; Waters 2795, Mississauga, Ontario, Canada) equipped with a 100 x 4 mm i.d.
279 Luna Omega PS C18 reversed-phase column (particle size 5 µm) using a water-acetonitrile gradient with a
280 constant 2 mmol L^{-1} concentration of ammonium acetate (0.6 $mL.min^{-1}$). Quantification of free 3-OH-C_{10} in
281 purified C_{10}-C_{10}, Rha-Rha-C_{10}-C_{10}, Rha-C_{10}-C_{10}, Rha-Rha-C_{14}-C_{14} or synthetic C_{10}-C_{10} were performed as
282 reported previously (34) and are presented in Supplementary table 2.
283
284 **Plant material and growth conditions.** *Arabidopsis thaliana* ecotype Col-0 was used as WT parent for all
285 experiments. Seeds from *fls2/efr1* (38, 39), *bak1*-5, *bkk1*-1, *bak1-5/bkk1-1* (40), *cerk1-2* (42), *bik1/pbl1* (41),
286 *rbohD, msl4/5/6/9/10* and *mca1/2* (51) were provided by C. Zipfel. Seeds from *sobir1*-12 and *sobir1*-13 (43)
287 were provided by F. Brunner (*Center for Plant Molecular Biology, University of Tübingen, Tübingen,*
288 PlantResponse™). Seeds from *sd1-29* (*lore-5*), Col-0^{AEQ} and *lore*-5^{AEQ} were provided by S. Ranf (45). *loh1* and
289 *fah1/2* seed (52) were provided by I. Feussner *(University of Göttingen, Germany)*. *dorn1*-1 seeds (44) were
290 obtained from NASC stock (SALK_042209). All mutants are in the Col-0 background. Plants were grown on soil
291 in growth chambers at 20°C, under 12 h light / 12 h dark regime with fluorescent light of 150 µmol m^{-2} s^{-1} and
292 60% relative humidity.
293
294 **Extracellular ROS production and calcium signaling.** ROS assays were performed on 4- to 6-week-old
295 *Arabidopsis* plants cultured on soil. Briefly, 5 mm long petiole sections were cut and placed in 150 µL of distilled
296 water overnight in 96 wells plate (PerkinElmer) (46). Then, the protocol was conducted as previously described
297 (82). Luminescence (relative light units, RLU) was measured every 2 min during 46 or 720 min with a Tecan
298 Infinite F200 PRO (or a TECAN CM SPARK for Supplementary figure 6), Tecan France. Total ROS production
299 was calculated by summing RLU measured between 4 to 46 or 4 to 720 minutes after treatment. Control was
300 realized on petioles of WT or mutant plants. $[Ca^{2+}]_{cyt}$ measurements were done as previously described (34).
301
302 **MAPK phosphorylation assays.** For MAPK phosphorylation assays, 3 leaf disks (9 mm diameter) were
303 collected from 4 to 6-week-old *Arabidopsis* plants grown on soil and incubated 8 h in distilled water. Leaf disks
304 were mock-treated or treated with different molecules. 15 min, 1 hour, and 3 hours after treatment, plant tissues
305 were frozen in liquid nitrogen. To extract proteins, 60 mg of leaf tissues were ground in a homogenizer Potter-
306 Elvehjem with 60 µL of extraction buffer (0.35 M Tris-HCl (pH 6.8), 30% (v/v) glycerol, 10% (v/v) SDS, 0.6 M
307 DTT, 0.012% (w/v) bromophenol blue). Total protein extracts were denatured for 7 min at 95°C, centrifuged at
308 11 000g for 5 min and 30 µL of supernatant were separated by 12% SDS-PAGE. Proteins were transferred onto

309 PVDF membranes for 10 min at 25 V using iBLOT gel transfer system (Invitrogen). After 30 min in 5% saturation
310 solution (50 g L^{-1} milk, TBS (137 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl), Tween20 0.05% (v/v)) and 3 times 5
311 min in 0.5% washing solution (5 g L^{-1} milk, TBS (137 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl), Tween 20 0.05%
312 (v/v)), membranes were incubated overnight with rabbit polyclonal primary antibodies against phospho-p44/42
313 MAPK (Erk1/2) (Cell Signaling, 1:2000) at 4°C. Then, membranes were washed 3 times 5 min with washing
314 solution and incubated 1 h with anti-rabbit IgG HRP-conjugated secondary antibodies (Bio-Rad, 1:3000) at room
315 temperature. Finally, washed membranes were developed with SuperSignal® West Femto using an odyssey
316 scanner (ODYSSEY® Fc Dual-Mode Imaging System, LI-COR). To normalize protein loading, membranes were
317 stripped 15 min with 0.25 M NaOH, blocked 30 min in 5% non-fat milk. Then, membranes were incubated at
318 room temperature for 1 h with plant monoclonal anti-actin primary antibodies (CusAb, 1:1000) and 1 h with anti-
319 mouse IgG HRP-conjugated secondary antibodies (Cell Signaling, 1:3000). Membranes were washed and
320 developed as described above.
321
322 **Conductivity assay.** The assay was performed as described previously (83), with few modifications. Eight leaf
323 discs of 6-mm-diameter were incubated in distilled water overnight. One disc was transferred into 1.5 mL tube
324 containing fresh distilled water and the corresponding elicitor concentration or ethanol for control. Conductivity
325 measurements (three to four replicates for each treatment) were then conducted using a B-771 LaquaTwin
326 (Horiba) conductivity meter.
327
328 ***Pseudomonas syringae* culture and disease resistance assays.** *Pseudomonas syringae* pv. *tomato* strain
329 DC3000 was grown at 28°C under stirring in King's B (KB) liquid medium supplemented with antibiotics: 50 µg
330 mL^{-1} of rifampicin and 50 µg mL^{-1} of kanamycin. For local protection assays, 15 seeds were sown per pot and
331 grown for 3 to 5 weeks in soil. Plants were sprayed with molecules or ethanol as control and were placed two
332 days in high humidity atmosphere before infections. Plants were inoculated by spraying the leaves with 3 mL of
333 a bacterial suspension at an optical density (OD_{600}) of 0.01 (0.025 % Silwet L-77, 10 mM $MgCl_2$). Quantification
334 (colony forming units) of *in planta* bacterial growth was performed 3 dpi. To this end, all plant leaves from the
335 same pot were harvested, weighed, and crushed in a mortar with 10 mL of 10 mM $MgCl_2$ and serial dilutions
336 were performed. For each dilution, 10 µL were dropped on KB plate supplemented with appropriate antibiotics.
337 Colony forming units (CFU) were counted after 2 days of incubation at 28°C. The number of bacteria per mg of
338 plants fresh mass was obtained with the formula:
339
340 $$\mathrm{CFU.mg^{-1}} = \frac{\left(\frac{N \times Vd}{Vi} \times 10^{(n-1)} \times 100\right)}{M}$$
341
342 with N equal to CFU number, Vi the volume depot on plate, Vd the total volume, n the dilution number and M
343 the plants fresh mass.
344
345 **References**

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Author contributions

J.C. and S.D. designed the research; R.S., J.C., A.K., T.G., M.T., S.V., S.D.C. performed the experiments; A.N. and E.D. purified and characterized HAAs and *B. glumae* RLs; M.C. and C.G. chemically synthetized HAAs; N.B., J.H., A.H. and J.H.R., purified *P. aeruginosa* RLs; C.D. and C.S. quantified mc-3-OH-FAs in all samples; R.S., J.C., S.C., F.M.G., F.B., S.R., E.D. and S.D. analyzed the data; R.S., J.C. and S.D. wrote the manuscript. M.O., J.H.R., A.H., T.H., C.Z., F.B., C.C., S.R and E.D contributed ideas, and critically revised the manuscript. All authors discussed the results and approved the manuscript.

Additional Information

Data availability

The authors declare that all data supporting the findings of this study can be found within the manuscript and its Supplementary Files. Additional data supporting the findings of this study are available from the corresponding authors upon request.

Competing financial interests

Technical University of Munich has filed a patent application to inventors A.K., C.D., T.H., and S.R. The authors declare no financial conflicts of interest in relation to this work.

All other author(s) declare no competing financial and/or non-financial interests.

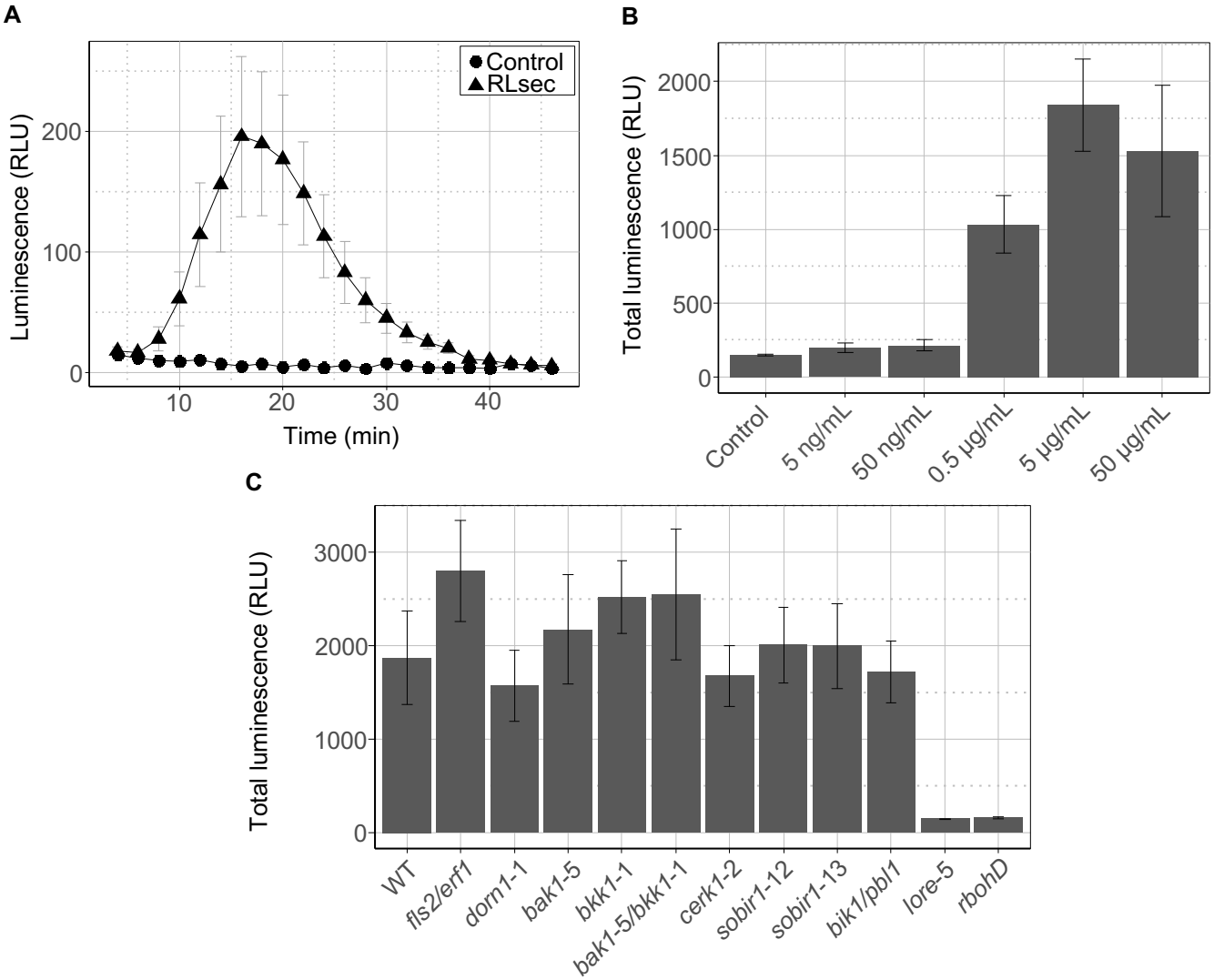


Fig. 1. RLsec activates LORE-dependent early immune-related responses in *Arabidopsis*. (A) Extracellular ROS production after treatment of WT leaf petioles with 50 µg/mL RLsec or EtOH as control. (B) Dose effect of RLsec on ROS production. ROS production measured after treatment of WT leaf petioles with the indicated concentrations of RLsec or EtOH as control. (C) ROS production measured after treatment of WT, *fls2/efr1*, *dorn1*-1, *bak1*-5, *bkk1*-1, *bak1*-5/*bkk1*-1, *cerk1*-2, *sobir1*-12, *sobir1*-13, *bik1/pbl1*, *lore*-5, or *rbohD* leaf petioles with 50 µg/mL RLsec. **(a,b,c)** Data are mean ± SEM (n = 6). Experiments have been realized three times with similar results.

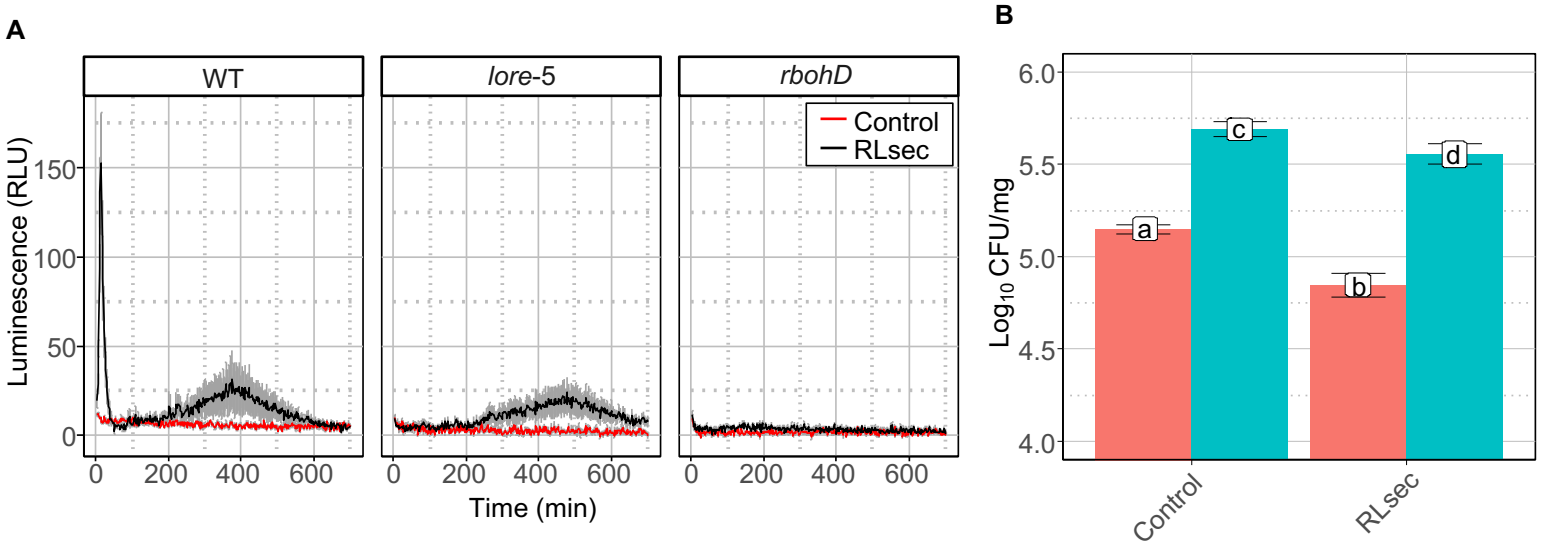


Fig. 2. RLsec activates LORE-independent responses in *Arabidopsis*. (A) Extracellular ROS production after treatment of WT, *lore*-5, and *rbohD* leaf petioles with 50 µg/mL RLsec or EtOH (Control). ROS production was monitored over 720 minutes. Data are mean ± SEM (n = 6). Experiments have been realized three times with similar results. (B) WT (red) and *lore*-5 (blue) *Arabidopsis* leaves were treated with 50 µg/mL RLsec or EtOH (control) 48 h before infection. *Pst* titers were measured at 3 d.p.i. Data are mean ± SD (n = 6, 5, 6, 6 (left to right)). Experiments have been realized twice with similar results. Letters represent results of pairwise Wilcoxon-Mann-Whitney statistic test with $P > 0.05$ (same letters) or $P \leq 0.05$ (different letters).

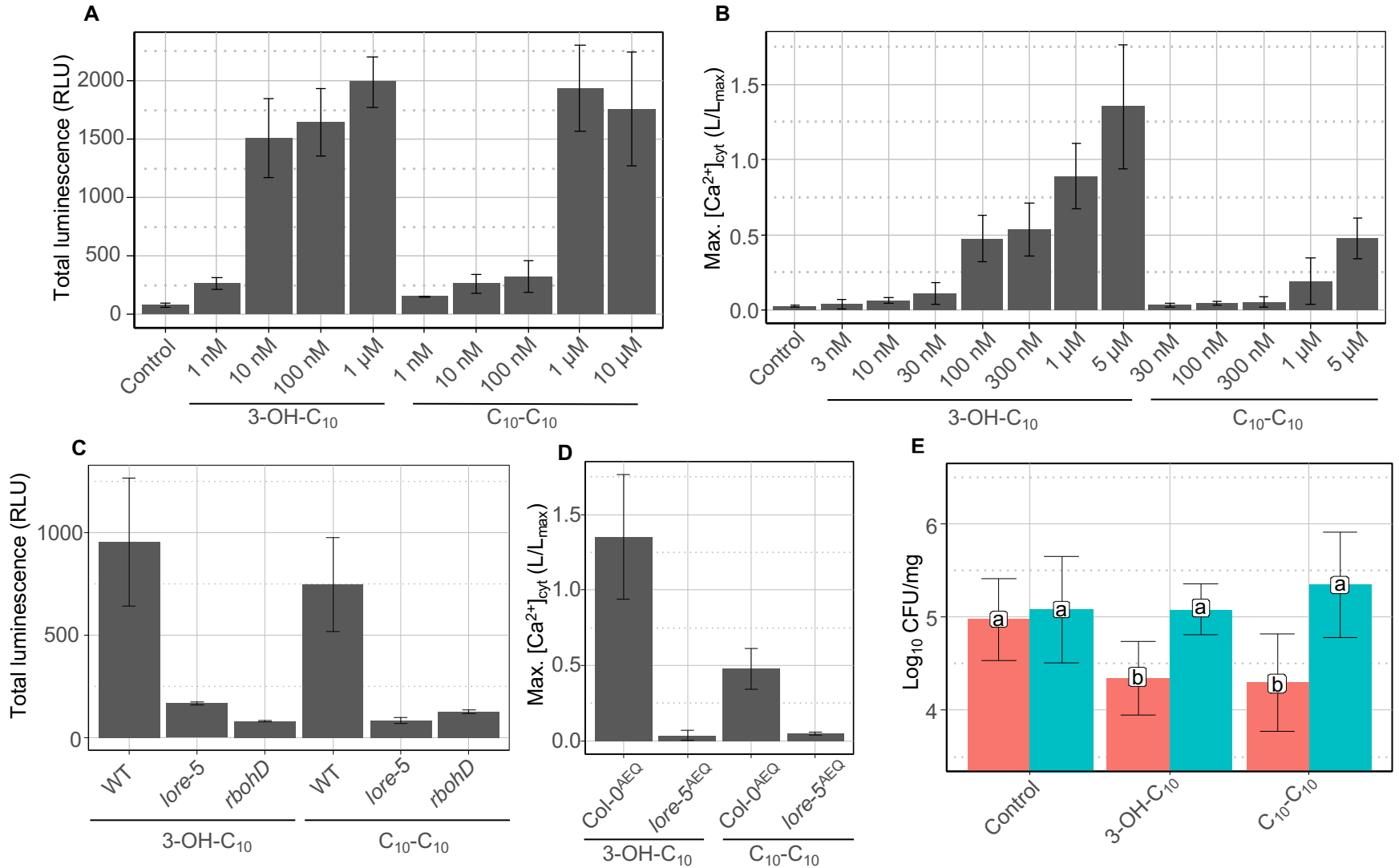


Fig. 3. Purified HAAs from *P. aeruginosa* trigger a LORE-dependent immune response in *Arabidopsis*. (A) Dose effect of 3-OH-C_{10} and C_{10}-C_{10} purified from *P. aeruginosa* on ROS production by WT leaf petioles. EtOH was used as negative control. Data are mean ± SEM (n = 6). Experiments have been realized twice with similar results. (B) Maximum (Max.) increases in $[Ca^{2+}]_{cyt}$ in *Arabidopsis* Col-0^{AEQ} seedlings treated with different concentrations of 3-OH-C_{10}, C_{10}-C_{10} purified from *P. aeruginosa* or MeOH as control. Data are mean ± SD (n = 3). Experiments have been realized twice with similar results. (C) ROS production measured after treatment of WT, *lore*-5, or *rbohD* leaf petioles with 10 µM 3-OH-C_{10}, 10 µM purified C_{10}-C_{10} or EtOH as control. Data are mean ± SEM (n = 6). Experiments have been realized three times with similar results. (D) Maximum (Max.) increases in $[Ca^{2+}]_{cyt}$ in *Arabidopsis* Col-0^{AEQ} and *lore*-5^{AEQ} seedlings treated with 5 µM 3-OH-C_{10} or purified C_{10}-C_{10}. Data are mean ± SD (n = 3). Experiments have been realized twice with similar results. For B and D, the same Col-0^{AEQ} 5µM data are presented (same experiments). (E) WT (red) and *lore*-5 (blue) *Arabidopsis* leaves were treated with 10 µM 3-OH-C_{10}, 10 µM purified C_{10}-C_{10} or EtOH (control) 48 h before infection. *Pst* titers were measured at 3 d.p.i. Data are mean ± SD (n = 27, 31, 38, 13, 30, 37 (left to right)). Experiments have been realized twice with similar results. Letters represent data of pairwise Wilcoxon-Mann-Whitney statistic test with $P > 0.05$ (same letters) or $P \leq 0.05$ (different letters).

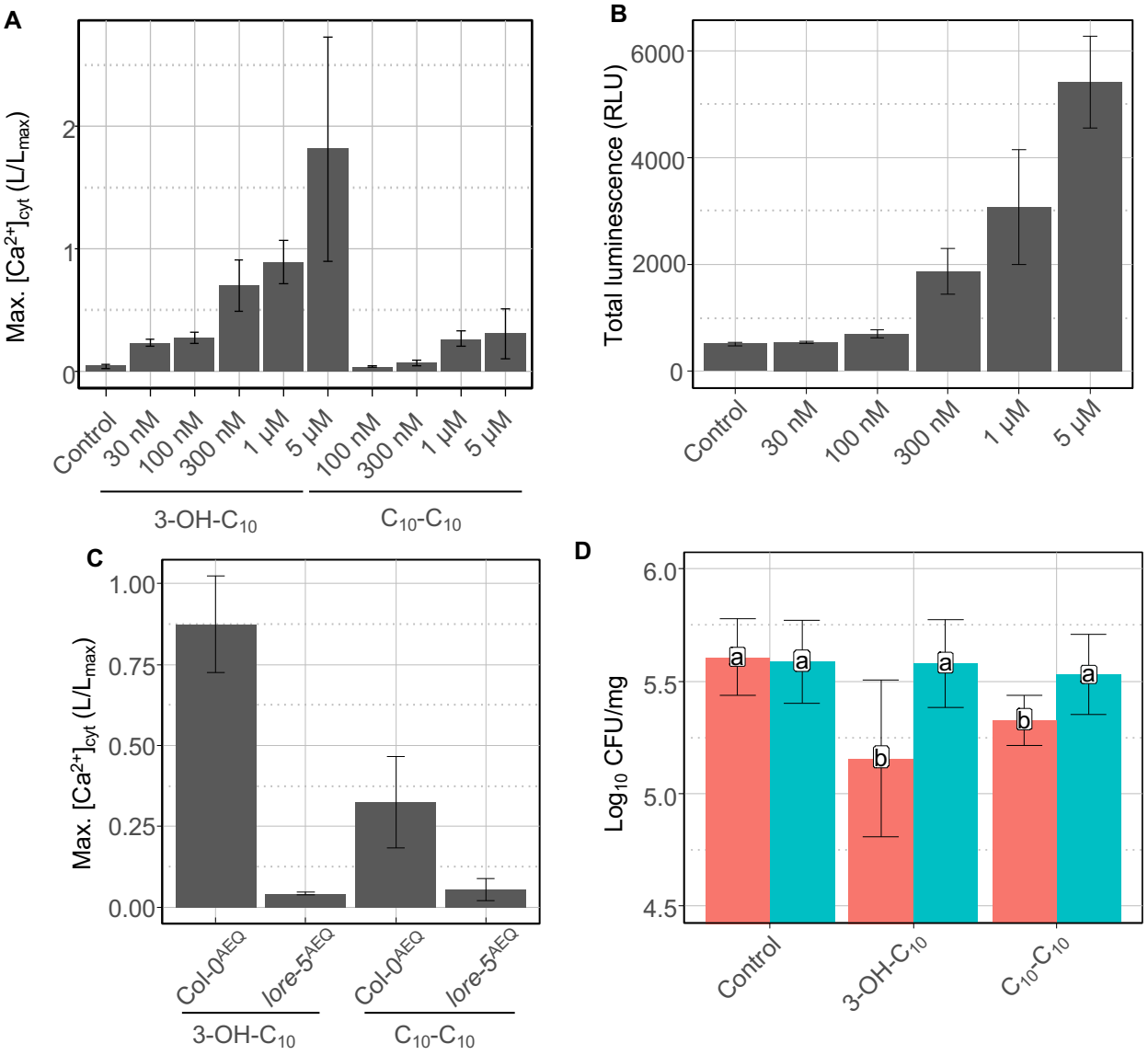


Fig. 4. Synthetic HAAs trigger a LORE-dependent immune response in *Arabidopsis*. (A) Maximum (Max.) increases in $[Ca^{2+}]_{cyt}$ in *Arabidopsis* Col-0^{AEQ} seedlings treated with different concentrations of 3-OH-C_{10}, synthetic C_{10}-C_{10} or MeOH. Data are mean ± SD (n = 3). Experiments have been realized twice with similar results. (B) Dose effect of synthetic C_{10}-C_{10} on ROS production by WT leaf petioles. EtOH was used as negative control. Data are mean ± SEM (n = 6). Experiments have been realized twice with similar results. (C) Maximum (Max.) increases in $[Ca^{2+}]_{cyt}$ in *Arabidopsis* Col-0^{AEQ} and *lore*-5^{AEQ} seedlings treated with 5 µM 3-OH-C_{10}, synthetic C_{10}-C_{10} or MeOH. Data are mean ± SD (n = 3). Experiments have been realized twice with similar results. (D) WT (red) and *lore*-5 (blue) *Arabidopsis* leaves were treated with 10 µM 3-OH-C_{10}, 10 µM synthetic C_{10}-C_{10}, or MeOH (control) 48 h before infection. *Pst* titers were measured at 3 d.p.i. Data are mean ± SD (n = 17, 21, 21, 30, 14, 30 (left to right)). Experiments have been realized twice with similar results. Letters represent data of pairwise Wilcoxon-Mann-Whitney statistic test with $P > 0.05$ (same letters) or $P \leq 0.05$ (different letters).

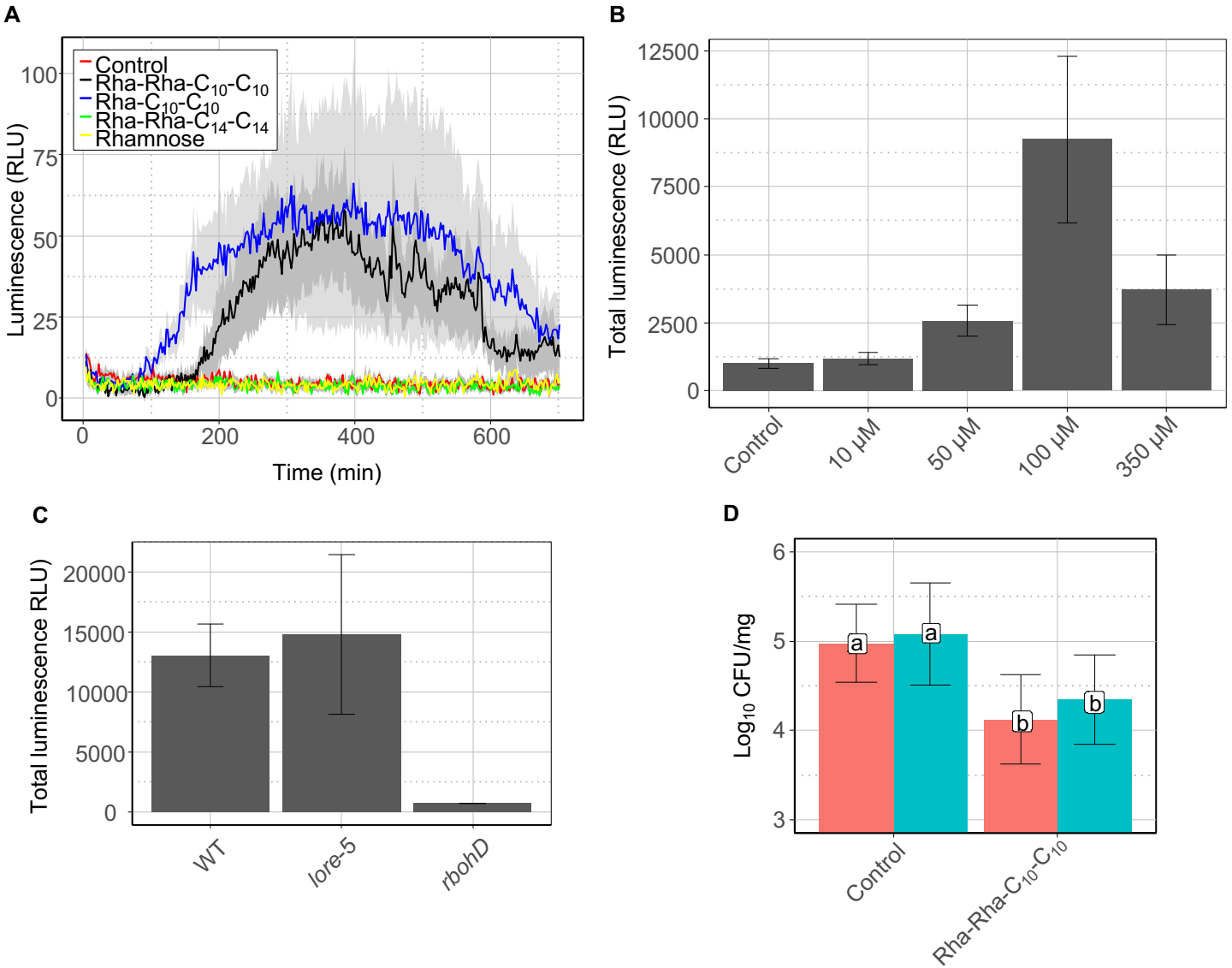


Fig. 5. Purified RLs trigger a LORE-independent *Arabidopsis* immune response. (A) Extracellular ROS production after treatment of WT leaf petioles with 100 µM RLs, 100 µM L-rhamnose, or EtOH (control). Data are mean ± SEM (n = 6). (B) Dose effect of Rha-Rha-C_{10}-C_{10} on ROS production. ROS production measured after treatment of WT leaf petioles with the indicated concentrations of Rha-Rha-C_{10}-C_{10} or EtOH (control). Data are mean ± SEM (n = 6). (C) ROS production measured after treatment of WT, *lore*-5, or *rbohD* leaf petioles with 100 µM Rha-Rha-C_{10}-C_{10}. Data are mean ± SEM (n = 6). (D) WT (red) and *lore-5* (blue) *Arabidopsis* leaves were treated with 10 µM Rha-Rha-C_{10}-C_{10} or EtOH (control) 48 h before infection. *Pst* titers were measured at 3 d.p.i. Data are mean ± SD (n = 27, 31, 30, 26 (left to right)). Letters represent data of pairwise Wilcoxon-Mann-Whitney statistic test with $P > 0.05$ (same letters) or $P \leq 0.05$ (different letters). (A-D) Experiments have been realized three times with similar results.

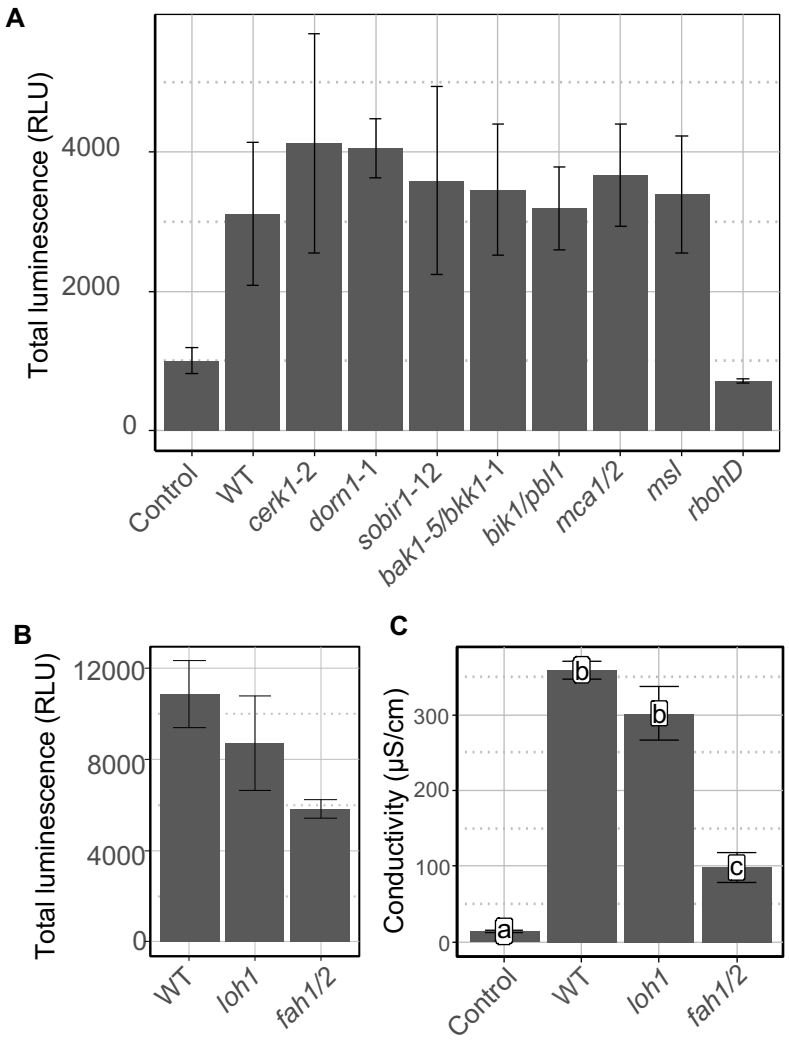
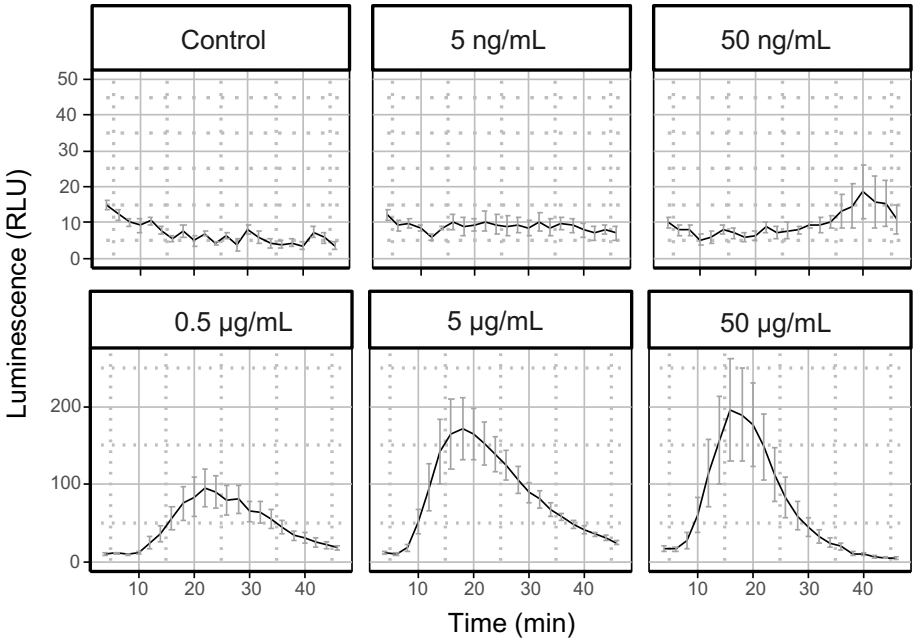
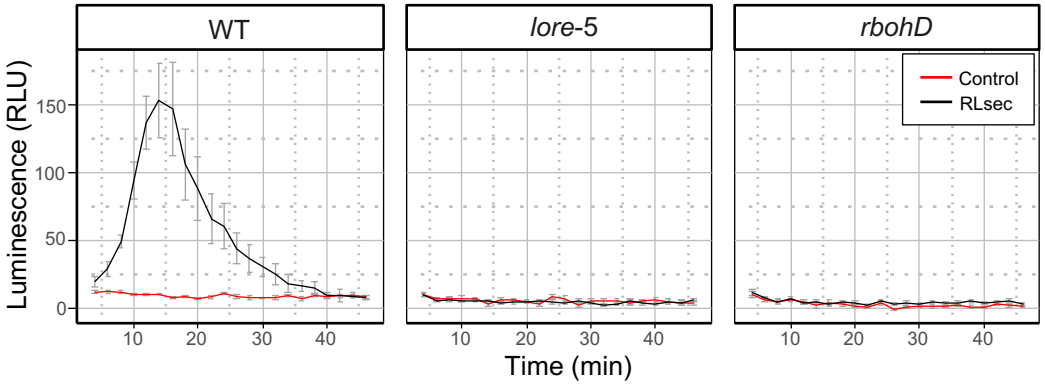


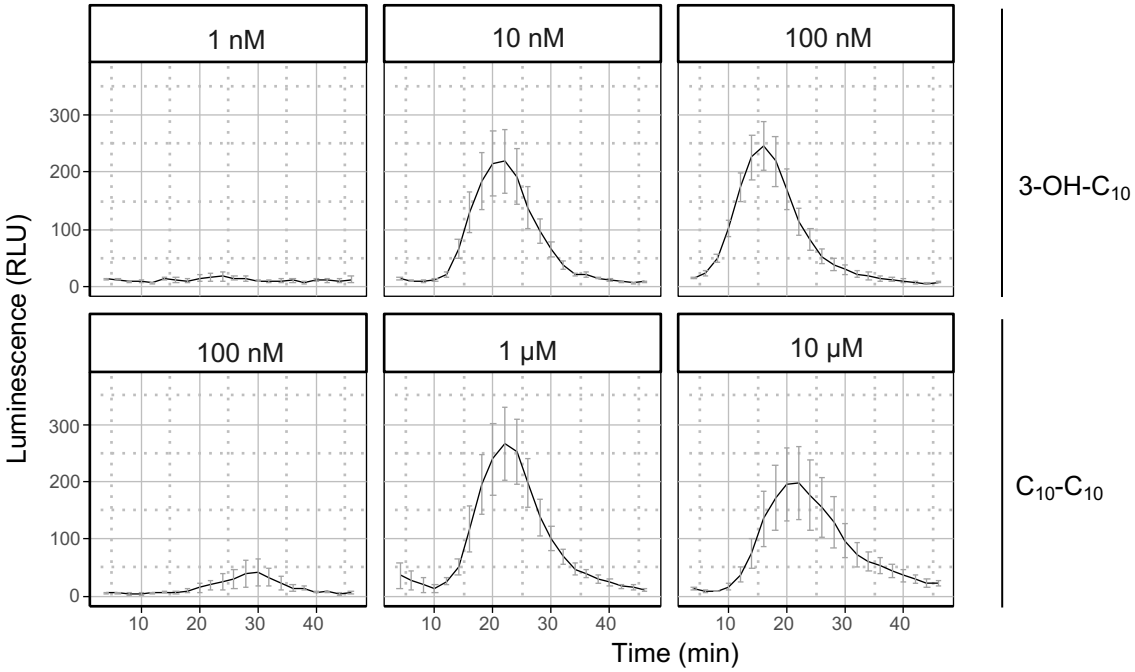
Fig. 6. RL perception is impacted by plasma membrane sphingolipid composition. Extracellular ROS production after treatment of (A) WT, *cerk1*-2, *dorn1*-1, *sobir1*-12, *bak1*-5/*bkk1*-1, *bik1/pbl1*, *mca1/2*, *msl4/5/6/9/10* (*msl*), or *rbohD,* and (B) WT, *loh1*, or *fah1/2 Arabidopsis* leaf petioles with 100 µM Rha-Rha-C_{10}-C_{10} or EtOH (control). Data are mean ± SEM (n = 6). Experiments have been realized three times with similar results. (C) Electrolyte leakage induced by 100 µM Rha-Rha-C_{10}-C_{10} or EtOH (Control) on WT, *loh1*, or *fah1/2 Arabidopsis* leaf discs 24h post treatment. Data are mean ± SEM (n = 6). Letters represent data of pairwise Wilcoxon-Mann-Whitney statistic test with $P > 0.05$ (same letters) or $P \leq 0.05$ (different letters). Experiments have been realized twice with similar results.



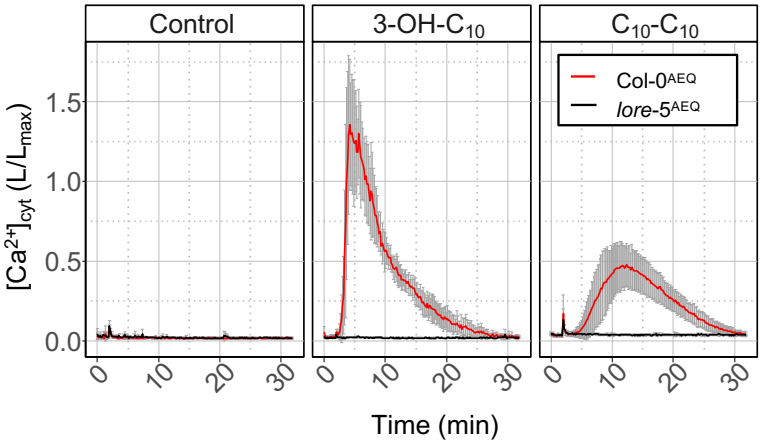
Supplementary fig. 1. RLsec dose effect on ROS. ROS production measured after treatment of WT leaf petioles with RLsec at the indicated concentrations or EtOH (control). Data are mean ± SEM (n = 6). Experiments have been realized three times with similar results. The data presented here as kinetic are from the same experiments illustrated in fig. 1B.



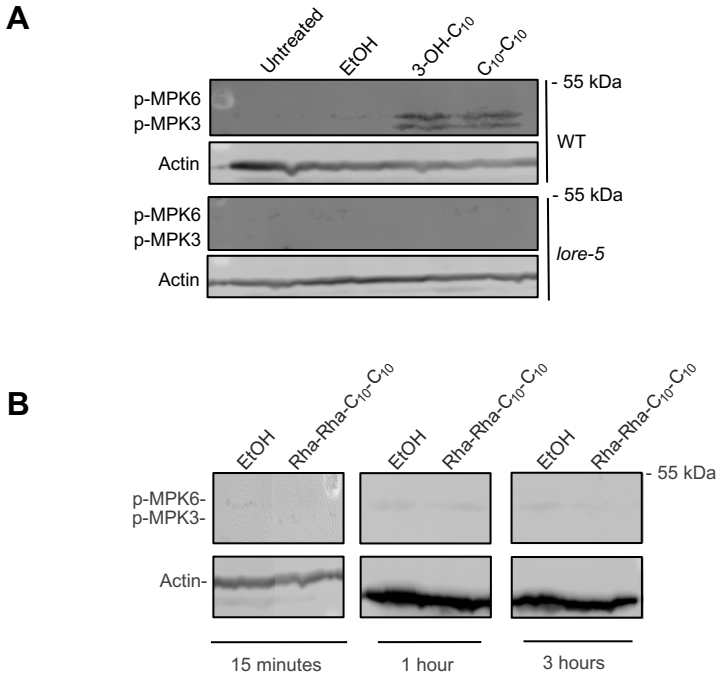
Supplementary fig. 2. RLsec induce early ROS production through LORE and RBOHD in *Arabidopsis*. Extracellular ROS production after treatment of WT, *lore*-5, or *rbohD* leaf petioles with 50 µg/mL RLsec or EtOH (control). Data are mean ± SEM (n = 6). Experiments have been realized three times with similar results. The data presented here as kinetic are from the same experiments illustrated in fig. 1C.



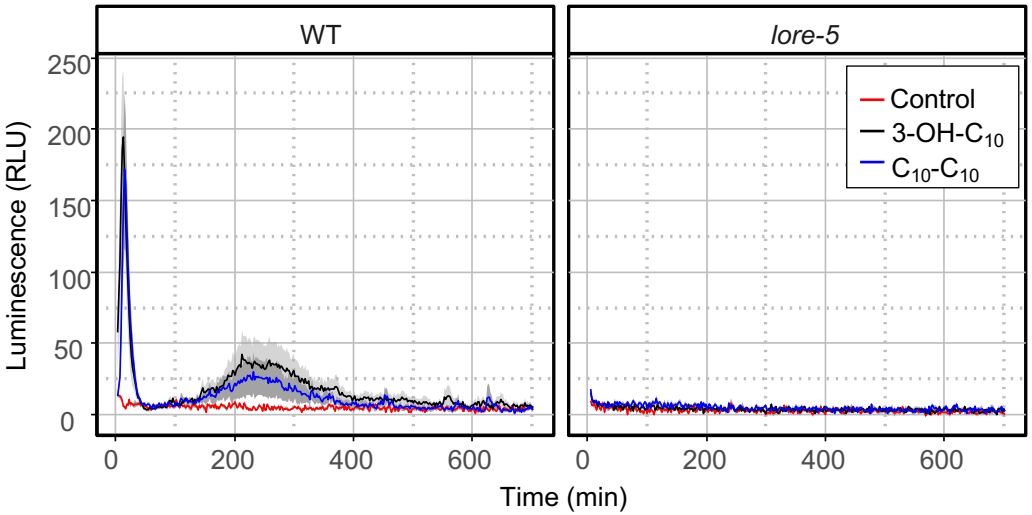
Supplementary fig. 3. Dose effect of 3-OH-C_{10} and C_{10}-C_{10} purified from *P. aeruginosa* on ROS production. ROS production measured after treatment of WT leaf petioles with the indicated concentrations of 3-OH-C_{10} and purified C_{10}-C_{10}. Data are mean ± SEM (n = 6). Experiments have been realized twice with similar results. The data presented here as kinetic are from the same experiments illustrated in fig. 3A.



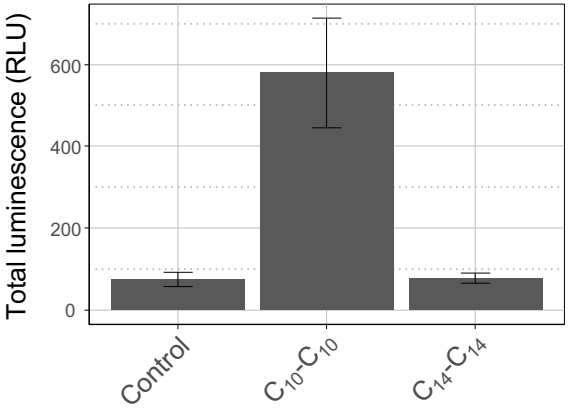
Supplementary fig. 4. Time course Ca^{2+} signaling. $[Ca^{2+}]_{cyt}$ over time in Col-0^{AEQ} and *lore*-5^{AEQ} seedlings after treatment with 5 µM purified C_{10}-C_{10}, 5 µM 3-OH-C_{10} or MeOH (control). Data are mean ± SD (n = 3). Experiments have been realized twice with similar results. The data presented here as kinetic are from the same experiments illustrated in fig. 3B.



Supplementary fig. 5. MAPK assay. Activation of MPK3 and MPK6 in (A) WT and *lore*-5 leaf disk 15 minutes after treatment with 10 µM 3-OH-C_{10}, 10 µM purified C_{10}-C_{10} or EtOH; (B) WT leaf disk 15 minutes, 1 hour, and 3 hours after treatment with 100 µM Rha-Rha-C_{10}-C_{10} or EtOH. Actin was used as loading control. Experiments have been realized twice with similar results.



Supplementary fig. 6. ROS production measured after treatment of WT or *lore*-5 leaf petioles with 10 µM 3-OH-C_{10}, 10 µM purified C_{10}-C_{10} or EtOH (control). Data are mean ± SEM (n = 6). Experiments have been realized twice with similar results.



Supplementary fig. 7. Chain length of HAAs impact *Arabidopsis* ROS immune response. ROS production measured after treatment of WT leaf petioles with 10 µM of purified C_{10}-C_{10} from *Pseudomonas aeruginosa,* C_{14}-C_{14} purified from *Burkholderia glumae* or with EtOH (control). Data are mean ± SEM (n = 6). Experiments have been realized three times with similar results.

Molecule	% (dry weight)
3-OH-C_8, 3-OH-C_{10}, 3-OH-C_{12}	**0.37**
HAAs	**3.75**
C_8-C_8	0.23
C_8-C_{10}	0.85
C_{10}-C_{10}	2.13
C_{10}-C_{12}	0.26
C_{12}-C_{12}	0
C_8-$C_{12:1}$	0.09
C_{10}-$C_{12:1}$	0.12
C_{12}-$C_{12:1}$	0.07
monorhamnolipids	**50.94**
Rha-C_8-C_8	0
Rha-C_8-C_{10}	5.28
Rha-C_{10}-C_{10}	37.61
Rha-C_{10}-C_{12}	3.53
Rha-C_{12}-C_{12}	0.06
Rha-C_8-$C_{12:1}$	0.72
Rha-C_{10}-$C_{12:1}$	3.55
Rha-C_{12}-$C_{12:1}$	0.19
dirhamnolipids	**44.94**
Rha-Rha-C_8-C_8	0.36
Rha-Rha-C_8-C_{10}	4.33
Rha-Rha-C_{10}-C_{10}	33.14
Rha-Rha-C_{10}-C_{12}	4.22
Rha-Rha-C_{12}-C_{12}	0.10
Rha-Rha-C_8-$C_{12:1}$	0.64
Rha-Rha-C_{10}-$C_{12:1}$	1.88
Rha-Rha-C_{12}-$C_{12:1}$	0.27

Supplementary table 1. RLsec composition. Distribution of congeners (percent) present in the lipidic secretome produced by *P. aeruginosa* (Jeneil, JBR-599, lot. #050629).

Molecules	Origin	Reference	Sample concentration (for quantification)	Free 3-OH-C_{10} concentration	Sample concentrations (for biological assays)	Concentration of 3-OH-C_{10} at the compound concentration used for the experiments	MTI in *Arabidopsis*	LORE-dependent
Mono-RL (Rha-C_{10}-C_{10})	*Pseudomonas aeruginosa* PA14	36	5 mM	0.28 µM	100 µM	5 nM	Yes	No
Di-RL (Rha-Rha-C_{10}-C_{10})	*Pseudomonas aeruginosa* PA14	36	5 mM	0.05 µM	10 µM to 350 µM	100 pM to 3 nM	Yes	No
Di-RL (Rha-Rha-C_{14}-C_{14})	*Burkholderia glumae*	53	nd	nd	100 µM	nd	No	No
C_{14}-C_{14}	*Burkholderia glumae*	this study	1 mM	<LOQ	10 µM	<LOQ	No	No
C_{10}-C_{10}	*Pseudomonas aeruginosa* PA14	this study	0.1 mM	0.09 µM	1 nM to 10 µM	0.9 pM to 9 nM	Yes	Yes
Synthetic C_{10}-C_{10}	Chemical synthesis (see Supplementary data 1 and 2)	this study	0.1 mM	<LOQ	30 nM to 10 µM	<LOQ	Yes	Yes

Supplementary table 2. Quantification of free 3-OH-C_{10} in HAA and RL samples.