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► To cite this version:

Sébastien Schoenaers, Hyun Kyung Lee, Martine Gonneau, Elvina Faucher, Thomas Levasseur, et al.. Rapid alkalization factor 22 has a structural and signalling role in root hair cell wall assembly. Nature Plants, 2024, 10.1038/s41477-024-01637-8 . hal-04500803

HAL Id: hal-04500803

<https://hal.science/hal-04500803v1>

Submitted on 12 Mar 2024

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1 A pectin-binding peptide with a structural and signaling role in the 2 assembly of the plant cell wall

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30 **Summary**

31 Pressurized cells with strong walls make up the hydrostatic skeleton of plants. Assembly and
32 expansion of such stressed walls depend on a family of secreted RAPID ALKALINIZATION FACTOR
33 (RALF) peptides which, curiously, bind both a membrane receptor complex and wall-localized
34 LEUCINE-RICH REPEAT EXTENSINS (LRXs) in a mutually exclusive way. Here we show that, in root
35 hairs, the RALF22 peptide has a dual structural and signaling role in cell expansion. Together with
36 LRX1, it directs the compaction of charged pectin polymers at the root hair tip into periodic
37 circumferential rings. Free RALF22 induces the formation of a complex with LORELEI-LIKE-GPI-
38 ANCHORED PROTEIN 1 (LLG1) and FERONIA (FER), triggering adaptive cellular responses. These
39 findings show how a peptide simultaneously functions as a structural component organizing cell wall
40 architecture and as a signaling molecule that regulates this process. This mechanism may also
41 underlie wall assembly and expansion in other plant cell types.

42

43 **Keywords:** RALF, cell wall, pectin, cell growth, CrRLK1L, LRX, root hair, FERONIA, cell wall integrity

44

45 Introduction

46 The pectic polysaccharides homogalacturonans (HGs), like glycosaminoglycans (GAGs) in the animal
47 extracellular matrix (ECM)¹, are abundant unbranched and charged polymers that play a critical role
48 in the control of the physico-chemical properties of the plant cell wall (CW)². These properties
49 include the remarkable ability to expand while simultaneously resisting the tensile forces imposed by
50 the turgor pressure on the CW².

51 Pectins are galacturonic acid-containing polymers, which, together with hemicelluloses (e.g.
52 xyloglucans) and (glyco)proteins, form the matrix that surrounds and connects cellulose microfibrils
53 in growing cells³. HGs form the most abundant class of pectins, reaching up to 50% of the total CW
54 polymer content⁴. They are synthesized in a highly methylesterified, uncharged form, and can be
55 demethylesterified by wall-associated pectin methylesterases (PMEs), thus generating random or
56 block-wise anionic charge patterns². *In vivo* studies have associated HG demethylesterification with
57 either the cessation or promotion of cell expansion, depending on the context⁵. Calcium (Ca²⁺)-
58 mediated crosslinking of polyanionic HG has been proposed as a driver for CW stiffening and growth
59 restriction^{6,7}. Growth promotion instead, has been proposed to occur upon the exchange of load-
60 bearing Ca²⁺ crosslinks with newly generated pectate, pectin swelling, or more complex scenarios
61 involving HG turnover or feedback signaling⁸⁻¹⁵. In any case, the physicochemical mechanisms in the
62 pectic CW underlying growth changes remain poorly understood so far.

63 In this context, it is interesting to note that the properties of the ECM in animals depend not only on
64 the intrinsic physical properties of the GAG polymers but also on the interaction of GAGs and their
65 core proteins with specific GAG binding proteins that modulate their structure¹⁶. For instance,
66 whereas GAGs and GAG-rich proteoglycans promote ECM swelling and generate ultrasoft matrices,
67 the presence of crosslinking proteins leads to water loss, compaction and rigidification of the same
68 matrices¹⁶. In addition, compaction can lead to the transition from a homogenous phase to
69 segregated microphases, as observed for instance for the formation of perineural nets, the porous
70 ECM that wraps neurons¹⁶.

71 In plants, some *in vitro* evidence shows the interaction of pectin with basic versions of extensins,
72 which are hydroxyproline-rich structural proteins that can form a crosslinked network in the CW.
73 While *in vivo* confirmation is still lacking, these interactions are believed to be partly established by
74 intermolecular di-isodityrosine oxidative crosslinks¹⁷⁻²⁰. Despite the similarities between plant HG and
75 animal GAG polymers, so far it is not known whether pectin-binding proteins contribute to the
76 modulation of CW properties in plants.

77 Here we show that RALF22, a member of the Rapid Alkalinisation Factor (RALF) family is a driver for
78 the structuration of the CW through its interaction with polyanionic pectin. RALF peptides were first
79 identified by their capacity to induce a rapid alkalinization of the growth medium and are part of a
80 family of 37 members in *Arabidopsis thaliana*^{21,22}. The well-studied peptide RALF23 binds to the GPI-
81 anchored protein LORELEI-LIKE-GPI-ANCHORED PROTEIN 1 (LLG1), thus triggering the formation of a
82 ternary complex with the *Catharanthus roseus* Receptor-Like Kinase1-Like (*CrRLK1L*) protein FERONIA
83 (FER), involved in immunity signaling²³. RALF4 and 19 are essential for the growth of pollen tubes, in
84 which they activate a module consisting of LORELEI-LIKE-GPI-ANCHORED PROTEIN 2 (LLG2), the
85 *CrRLK1L* proteins BUDDHAS PAPER SEAL 1 (BUPS1) and ANXUR (ANX1), the cytosolic kinase MARIS
86 and the Ca²⁺ channel of the MILDEW RESISTANCE LOCUS O (MLO) family²⁴⁻²⁷. Related pathways are
87 required for sperm release from pollen tubes, CW integrity control, stress responses or
88 mechanosensing²⁸. Interestingly, several RALF peptides also tightly bind CW proteins of the LEUCINE-
89 RICH REPEAT EXTENSIN (LRX) family, with distinct and mutually exclusive binding modes to the LLG-
90 *CrRLK1L* signaling system^{27,29-31}. LRXs are dimeric CW proteins, essential for growth, with a RALF-
91 binding LRR domain and a C-terminal extensin domain rich in crosslinkable tyrosines, but without
92 patches of basic residues that are found in pectin-binding extensins^{29,32}.

93 In this study, we used *Arabidopsis* root hairs (RHs) as a model system to study RALF-mediated pectin
94 structuration and the biological function underlying the existence of two distinct classes of RALF-
95 binding proteins. RHs show highly polarized apical growth that can display oscillations in growth rate
96 and a number of other parameters, such as cytosolic Ca^{2+} levels, CW pectin charge, Reactive Oxygen
97 Species (ROS) levels and cytosolic and apoplastic pH^{33} . This suggests that RH growth is regulated by
98 processes that require, or are optimized by temporal synchronization, eventually leading to the
99 emergence of periodicity in CW modifying activities. For instance, processive pectin
100 demethylesterification is favored at neutral pH, whereas the remodeling of the cellulose-xyloglucan
101 network by the CW-loosening expansins occurs at low $\text{pH}^{34,35}$.

102 We show that a periodic CW architecture emerges in RHs from the charge-dependent compaction of
103 polyanionic pectin through its binding to the CW protein complexes LRX1/2-RALF22. In the absence
104 of RALF22 or LRX1/2, the CW architecture is severely perturbed, leading to a frequent loss of cell
105 integrity. Furthermore, when not incorporated into the CW, the same peptide has a signaling role
106 through the formation of a membrane ternary complex with LLG1-FER, which induces cellular
107 changes that influence CW assembly and growth.

108 Results

109 RALF22 is required for root hair growth

110 To study RALF-regulated cell expansion, we screened publicly available transcriptome data for RH-
111 expressed *RALF(s)*. From the seven *RALF(s)* that had the highest expression in the primary root (Fig.
112 S1A) only *RALF22* transcripts were enriched in RH cells, as shown by single cell RNAseq data (Fig. S1B-
113 C), and its expression correlated with the trichoblast developmental stage (Fig. S1D)³⁶. Using CRISPR-
114 Cas9, we generated a *RALF22* loss-of-function line by a 182-nucleotides deletion 51 nucleotides
115 downstream of the start codon (*ralf22-1*; Fig. 1A). A second loss-of-function allele was identified from
116 the GabiKat seed repository (*ralf22-2*; GK_293H09; Fig. 1A). Both lines displayed a distinct short and
117 bulged RH phenotype with frequent cell bursting, which was fully complemented by re-introducing
118 the wild-type *pRALF22::RALF22* sequence into the mutant genome (Fig. 1B-C; movie 1).
119 Transcriptional reporter lines (Col-0 x *pRALF22::GFP*) accumulated GFP in RH cells only (Fig. 1D), and
120 showed that *RALF22* transcription commenced during RH bulge formation, peaked during tip growth
121 and ceased upon RH maturation (Fig. 1D). These data show that *RALF22* is essential for normal RH
122 morphogenesis.

123 RALF22 has FERONIA-dependent and FERONIA-independent effects on root hair 124 growth

125 To study *RALF22* function, we investigated whether *RALF22* is a RH-specific CrRLK1L ligand,
126 similar to other CrRLK1L-binding RALF peptides^{24,37,38}. Like other RALFs, *RALF22* contains a
127 propeptide sequence that is cleaved off by subtilisin-like serine proteases during protein
128 maturation^{39,40}. The mature *RALF22* protein harbors two disulfide cysteine-bridges and a
129 conserved YISY motif that is critical for receptor binding (Fig. 2A)^{23,39}. To date, two CrRLK1L
130 receptor-like kinases (ERU, FER) and the FER-RALF23 co-receptor LLG1, were shown to be
131 required for RH morphogenesis⁴¹⁻⁴³. We recombinantly expressed LLG1 and the ERU and FER
132 ectodomains (ERU_{ecd}, FER_{ecd}) in insect cells and purified them for interaction analysis (Fig. S2A-B).
133 In addition, we chemically synthesized *RALF22*. We then quantified the binding affinities
134 amongst all possible protein combinations (Fig. S2C) using microscale thermophoresis (MST).
135 While *RALF22* interacted with LLG1 with a dissociation constant (Kd) of $6.09 \pm 1.13 \mu\text{M}$ (Fig. 2B), it
136 did not directly interact with ERU_{ecd} or FER_{ecd} alone (Fig. S2C). Preincubated LLG1-RALF22,
137 however, formed a high affinity ($118.02 \pm 59.01 \text{nM}$) ternary complex with FER_{ecd}, but not with
138 ERU_{ecd} (Fig. 2C-D, Fig. S2C). Next, we substituted both tyrosines with alanines in the conserved
139 *RALF22* YISY motif (*RALF22*^{Y75A,Y78A}) that mediates receptor binding²³. Compared to wild type
140 *RALF22*, we observed a 4.7-fold decrease in the affinity of *RALF22*^{Y75A,Y78A} for LLG1

141 (28.48±2.43μM) (Fig. 2B). In addition, LLG1-RALF22^{Y75A,Y78A} no longer formed a ternary complex
142 with FER_{ecd} (Fig. 2C). Together, these results show that RALF22, like RALF23²³, binds to LLG1 and
143 nucleates the formation of a ternary LLG1-RALF22-FER_{ecd} complex.

144 Next, we explored the *in vivo* relevance of this interaction for RH growth. We optimized a
145 protocol that allowed us to reproducibly treat growing RHs while imaging key parameters
146 related to RH elongation, namely oscillations in growth rate, extracellular pH (pH_{ext}) and
147 intracellular calcium ([Ca²⁺]_{cyt}). To this end, we grew Col-0 and FERONIA loss-of-function (*fer-4*)
148 seedlings expressing the cytosolic Ca²⁺ indicator GCaMP3⁴⁴ in microfluidic chips in the presence
149 of FITC (pH-sensitive) and TRITC (pH-insensitive) coupled to 110kDa and 20kDa neutral dextran,
150 respectively. In animal systems, FITC or TRITC coupled to dextrans of different sizes are
151 commonly used for permeability studies^{45,46}. Here, the large dextran molecules restrict both
152 fluorophores to the extracellular medium and the FITC/TRITC ratio provides a ratiometric
153 quantification of the pH_{ext} (Fig. S3A). Using confocal microscopy, we monitored Col-0 and *fer-4*
154 RHs before and after administration of 5μM RALF22. During steady-state growth, both Col-0 and
155 *fer-4* RHs displayed oscillations in growth rate, [Ca²⁺]_{cyt} and pH_{ext} (Fig. 2E, F). Upon RALF22
156 treatment, Col-0 RHs immediately stopped growing (Fig. 2E, G). This growth arrest was
157 accompanied by a transient spike in [Ca²⁺]_{cyt} (Fig. 2E, H) and an increase in pH_{ext} (Fig. 2E, I), which
158 rapidly spread from tip to shank (Fig. S3B-D). The pH remained high over the next 6 min (Fig. 2E,
159 movie 2). Surprisingly, RALF22 treatment induced a clear migration and accumulation of both
160 dextran-coupled TRITC and FITC fluorophores into the CW, at a comparable rate, suggesting a
161 change in the physicochemical properties of the CW (Fig. 2E, J, movie 2). Upon RALF22
162 treatment, *fer-4* RHs showed a mild growth inhibition and an increase in [Ca²⁺]_{cyt} and pH_{ext} (Fig.
163 2F-I). In striking contrast to Col-0 RHs the extracellular alkalization did not propagate towards
164 the shank (Fig. S3B, E), while growth rate, [Ca²⁺]_{cyt} and pH_{ext} oscillations recovered rapidly (Fig.
165 2F-I, movie 2). Crucially, the RALF22-induced migration and accumulation of dextran-coupled
166 TRITC and FITC into the CW was still apparent in *fer-4* RHs (Fig. 2F, J; movie 2), suggesting that
167 besides its FER-dependent signaling role, RALF22 treatment has an additional FER-independent
168 effect on the physicochemical properties of the CW.

169 RALF22 interacts with demethylesterified homogalacturonan and induces its 170 compaction

171 To understand this FER-independent effect of RALF22 on the CW, it is interesting to note that
172 mature RALF22 is a positively charged protein (isoelectric point = 10.53) (Fig. 3A), which
173 potentially interacts electrostatically with poly-anionic demethylesterified homogalacturonan
174 (HG) in the CW. We used MST to study the interaction between RALF22 and a fully
175 demethylesterified oligogalacturonide, with a degree of polymerization between 7 and 13 (OG7-
176 13)⁴⁷. We observed a robust interaction with a K_d of 3.03±0.53μM (Fig. 3B). The charge
177 dependency of the interaction is suggested by the 3.7-fold reduction in affinity (K_d =
178 11.1±2.65μM) for a mutant peptide RALF22^{R82A,R90A,R100A} in which 3 charged arginines were
179 replaced by neutral alanine residues (Fig. 3B). To further explore this interaction, we used Quartz
180 Crystal Microbalance with Dissipation monitoring (QCM-D). This technique measures the mass
181 and viscoelasticity of polymer layers deposited on gold-coated oscillating quartz crystals, where
182 a decrease in oscillation frequency (ΔF) linearly correlates with a mass increase and a decrease in
183 the dissipation time of the oscillation after interruption of the current (ΔD) correlates with an
184 increase in viscoelasticity of the layer (Fig. 3C)⁴⁸. In our experimental setup, we first created a
185 pectin layer on the quartz surface (Fig. S4A, B). To this end, the gold-coated quartz, which
186 previously had been spin-coated with a positively charged polymer (poly-allylamine
187 hydrochloride, PAH), was placed in a flow cell and a pectin solution was flown over the surface
188 with a constant flow rate, while monitoring ΔF and ΔD (Fig. S4A, B). As expected, an elastic

189 pectin layer was formed as shown by the decrease in ΔF_{pectin} and increase in ΔD_{pectin} , both for
190 pectin with a degree of methylesterification (DM) of 75% (DM75, Fig. S4A) and 31% (DM31, Fig.
191 S4B; see Fig. S4C, D for a detailed characterization of the pectin preparations). After a washing
192 step, RALF22 was applied to the DM75 pectin layer (Fig. 3D, Fig. S4A). Unexpectedly, this showed
193 a mass decrease (seen as an increase in ΔF_{RALF22} corresponding to a $9.60 \pm 2.22\%$ loss of the mass
194 of the hydrated pectin layer, $n=14$) and a concomitant increase in stiffness (seen as a decrease in
195 ΔD_{RALF22} corresponding to a $34.05 \pm 6.63\%$ decrease in viscoelasticity of the pectin layer, $n=14$)
196 (Fig. 3D, G). These results indicate that RALF22 interacts with pectin, but also induces water loss
197 from the pectin layer, the mass of which exceeds the gain in mass due the binding of the 5.6 kDa
198 RALF22 peptide. This causes stiffening of the layer, which is expected for the formation of a
199 polyelectrolyte complex where the neutralization of the charges causes dewatering^{49,50}. This
200 interaction was stable under the experimental conditions used since no important changes in ΔF
201 and ΔD were observed during the subsequent washing step (Fig. 3D, Fig. S4A). The charge
202 dependence of the interaction was confirmed by the increased dewatering capacity of RALF22
203 on pectin with a lower degree of methylesterification (DM31; increase in ΔF_{RALF22} of
204 $38.98 \pm 4.27\%$, decrease in ΔD_{RALF22} of $94.13 \pm 8.36\%$, $n=15$) (Fig. 3E, G, Fig. S4B) as well as the
205 strongly reduced dewatering capacity of mutant peptide RALF22^{R82A,R90A,R100A} (increase in ΔF of
206 $3.53 \pm 0.24\%$, decrease in ΔD of $13.11 \pm 1.64\%$, $n=2$) relative to wild-type RALF22 (Fig. 3F, G). The
207 interaction with fully methylesterified HG could not be assessed using this technique since such
208 polymers do not bind the PAH layer. Together, these results show that, *in vitro*, RALF22 binds
209 and dewateres pectin in a charge-dependent manner. The formation of such a polyelectrolyte
210 complex not only creates denser zones in the gel, but also augments the porosity of the non-
211 compacted zones^{16,49,50}. An analogous process is thought to underlie the formation of perineural
212 nets, the porous aggregated matrix wrapped around the surface of neurons, which is formed
213 through the interaction between charged GAGs and crosslinking proteins¹⁶. Similar RALF22-
214 induced physicochemical changes (pectin charge neutralization, water loss and porosity
215 increase) could account for the FER-independent accumulation of FITC- and TRITC-dextran in the
216 CW of growing RHs (Fig. 2E, F, J; movie 2).

217 RALF22 forms periodic circumferential rings in the root hair cell wall

218 To investigate how endogenous RALF22 regulates cell expansion, we generated *ralf22-2* lines
219 expressing mCherry-tagged mature RALF22 (*ralf22-2 x pRALF22::mCherry-RALF22_{mature}*; see Star
220 Methods). *Ralf22-2 x pRALF22::mCherry-RALF22_{mature}* seedlings had RHs that were
221 indistinguishable from Col-0, showing that the tagged protein is fully functional (Fig. 4A, B).
222 Surprisingly, mCherry-RALF22 accumulated in the RH CW, where it formed regularly spaced
223 microdomains (Fig. 4C), which remained immobile throughout RH development (Fig. 4C, D,
224 movie 3A). These microdomains arise at the RH tip, the exclusive site of RALF22 secretion (Fig.
225 4E, movie 3B). Indeed, using Fluorescence Recovery After Photobleaching (FRAP) we found that
226 mCherry-RALF22 fluorescence rapidly recovered at the tip, but did not recover subapically (Fig.
227 4F, movie 4). At the growing apex, *de novo* mCherry-RALF22 deposition oscillated in antiphase
228 with the growth rate oscillations, illustrating that temporal growth rate periodicity is translated
229 into spatial periodicity in RALF22 microdomain formation in the CW (Fig. 4G). Closer inspection
230 of the mCherry-RALF22 microdomains, using spinning disk microscopy, revealed that they
231 represent regularly spaced rings surrounding the RH's tubular structure (Fig. 4H). These rings
232 first appear at the tip, after which they circumferentially expand 11.5 ± 0.9 -fold ($n=8$) in the
233 expanding dome.

234 To better understand this localization pattern, we compared the spatial periodicity of mCherry-
235 RALF22 rings along the RH's longitudinal axis with the temporal periodicity in RH growth rate
236 (Fig. S5A-D). Based on wavelet transformation of the longitudinal fluorescence pattern present in
237 the mCherry-RALF22 kymographs (e.g. Fig. 4D) the average RALF22 ring spacing was 1.34 ± 0.08

238 μm (Fig. S5A, B). Interestingly, for the same RHs, a single growth rate period ($30.47 \pm 0.93\text{s}$ at
239 $2.15 \pm 0.06 \mu\text{m} \cdot \text{min}^{-1}$) attributed to an average RH length gain which was 1.21-fold lower
240 ($1.09 \pm 0.04 \mu\text{m} \cdot \text{period}^{-1}$; Fig. S4C-E), suggesting that the spacing between RALF22 rings increases
241 when moving from tip to shank. We then used wavelet analysis to compare the mCherry-RALF22
242 ring spacing in the expanding dome and along the RH shank (Fig. 4I, Fig. S5F-K). Indeed, whilst
243 the spacing in the tip was $0.94 \pm 0.08 \mu\text{m}$, shank-localized rings were spaced 1.37-fold further
244 apart at $1.29 \pm 0.08 \mu\text{m}$ (Fig. 4I, Fig. S5J, K).

245 Together, these data show that, during RH growth, the temporal periodicity in growth rate
246 translates into the spatial periodicity in RALF22 ring formation in the CW. In addition, wall
247 expansion over the surface of the dome was highly anisotropic as shown by the 8.4x larger
248 circumferential strain (11.5-fold increase) of the rings relative to the meridional (from tip to
249 shank) strain (1.37-fold increase).

250 RALF22 forms a complex with LRX1 in root hair cell walls

251 The periodic arrangement of RALF22 in the RH CW suggests that it could associate with other
252 CW epitopes with a similar ring-like organization. Interestingly, RALF-mediated regulation of CW
253 integrity depends on two distinct receptor families, the biological relevance of which is unclear.
254 Hence, RALF peptides have previously been shown to bind CW proteins of the LEUCINE-RICH
255 REPEAT EXTENSIN (LRX) family, with distinct and mutually exclusive binding modes to the LLG-
256 CrRLK1L signaling system^{23,27,29-31}. In RHs, LRX1 and LRX2 have partially redundant roles in
257 sustaining RH growth⁵¹. The RH phenotype of the *lrx1-1/2-1* loss-of-function mutant is
258 indistinguishable from *ralf22*, suggesting that they could be functionally related (Fig. 5A, B,
259 movie 5). To investigate the plausibility of a RALF22-LRX1/2 complex, we generated a peptide
260 docking model using the crystal structure of the LRR domain of LRX2 (LRX2_{LRR}, PDB: 6QXP)²⁹ and
261 an AlphaFold2 model of the corresponding LRX1_{LRR} domain (Fig. 5C). RALF22_{mature} was modelled
262 using the crystal structure of RALF4 (PDB: 6TME) as a reference. Similar to the LRX8_{LRR}-RALF4
263 complex²⁹, we found that the conserved binding pockets of LRX2_{LRR} and LRX1_{LRR} could
264 accommodate RALF22_{mature} (Fig. 5C, LRX2_{LRR}-RALF22; total energy score = $-66.43 \text{ kcal} \cdot \text{mol}^{-1}$; root
265 mean square deviation = 0.760 \AA). LRX1_{LRR}-RALF22 and LRX2_{LRR}-RALF22 dimeric complexes could
266 indeed be purified from insect cells that co-expressed LRX1_{LRR} or LRX2_{LRR} with RALF22 (Fig. 5D).
267 These complexes were extremely stable as shown by the inability of LRX1_{LRR}-RALF22 to dissociate
268 at pH 2.0 (Fig. 5D, E) and their 92.3°C (LRX1_{LRR}-RALF22) and 83.8°C (LRX2_{LRR}-RALF22) dissociation
269 temperatures in thermal shift assays (Fig. 5F). These results suggest that the LRX1_{LRR}-RALF22 and
270 LRX2_{LRR}-RALF22 are bona fide complexes with even higher stability than the previously described
271 LRX8_{LRR}-RALF4 pollen expressed complex²⁹.

272 To study the LRX1-RALF22 interaction *in planta*, we introduced *pRALF22::mCherry-RALF22_{mature}*
273 into *lrx1-1* plants that express a functional cMyc-tagged LRX1 (*lrx1-1 x pLRX1::cMyc-LRX1*⁵²).
274 cMyc-LRX1 also formed rings perpendicular to the RH's longitudinal axis, that colocalized to a
275 great extent with mCherry-RALF22_{mature} (Fig. 5G, H; Costes p-value > 0.95; thresholded Manders'
276 coefficient (tM)_{mCherry-RALF22@cMyc-LRX1} = 0.71 ± 0.22 ; tM_{cMyc-LRX1@mCherry-RALF22} = 0.70 ± 0.21 ; n = 11). These
277 data are consistent with the presence of LRX1-RALF22 complexes *in planta* but are expected to
278 be incomplete given the presence of endogenous RALF22 and LRX2. Together, these data show
279 that RALF22 adopts a periodic pattern in the RH CW at least in part as an LRX1-RALF22 complex.

280 LRX1-RALF22 also binds and compacts demethylesterified homogalacturonan

281 Next, we investigated whether pectin binding is preserved in the LRX1_{LRR}-RALF22 complex. A
282 possible pectin interaction is suggested by the 3D crystal structure of the LRX8_{LRR}-RALF4 dimer, in
283 which RALF4 displays a highly positively-charged and surface-exposed patch of amino acids²⁹.
284 Homology modeling shows that this is also true for the LRX1_{LRR}-RALF22 complex, albeit with a
285 less pronounced surface charge compared to LRX8_{LRR}-RALF4 (Fig. 5C), comprising the three

286 arginines (R82, R90, R100) that are critical for free RALF22-pectin binding (Fig. 3B, F, G, Fig. 5C).
287 QCM-D indeed showed an interaction of the LRX1_{LRR}-RALF22 complex with DM75 and DM31
288 pectin layers (Fig. 5I-K). In contrast to free RALF22 however, we observed a charge-dependent
289 mass increase (decrease in $\Delta F_{\text{LRX1-RALF22}}$ for DM31 of $278.21 \pm 26.64\%$, $n=11$; decrease in $\Delta F_{\text{LRX1-}}$
290 RALF22 for DM75 of $98.62 \pm 31.52\%$, $n=6$) (Fig. 5I-K). Together with the mass increase, we observed
291 a decrease of the viscoelasticity ($\Delta D_{\text{LRX1-RALF22}}$ decrease for DM31 of $48.02 \pm 15.06\%$, $n=11$; $\Delta D_{\text{LRX1-}}$
292 RALF22 decrease for DM75 of $22.54 \pm 7.37\%$, $n=6$), indicating that LRX1_{LRR}-RALF22, like free RALF22,
293 promoted the stiffening of the pectin layers (Fig. 5K). Concerning the LRX1_{LRR}-RALF22-dependent
294 mass increase, as opposed to the mass decrease observed upon free RALF22 supply, this is most
295 likely due to the much higher mass of the LRX1_{LRR}-RALF22 dimer (94.18kDa) compared to the
296 mass of RALF22 alone (5.6kDa), which most likely exceeds the mass loss due to pectin
297 dewatering. The observed decrease in ΔD is presumably the resultant of the stiffening effect of
298 the LRX1_{LRR}-RALF22-pectin interaction and the decrease in viscoelasticity due to the addition of
299 extra mass to the layer. More detailed studies are needed to quantify these different effects.
300 Nevertheless, these results show that LRX1_{LRR}-RALF22, like free RALF22, is able to bind and
301 stiffen pectin in a charge-dependent manner.

302 RALF22 is required for the self-assembly of pectic homogalacturonan

303 Given the charge-dependence of the interaction of RALF22 and LRX1-RALF22 with pectin, pectin
304 demethylesterification is expected to be critical for the assembly and expansion of the RH CW.
305 To investigate this, we first supplemented growing RHs with the PME inhibitor EGCG, while
306 imaging $[\text{Ca}^{2+}]_{\text{cyt}}$, pH_{ext} and CW charge (Fig. S6; movie 6). EGCG treatment induced a short
307 decrease in growth rate, followed by a transient growth spurt and a transition from longitudinal
308 to radial expansion (=swelling) at the tip (Fig. S6A; movie 6). This response was accompanied by
309 a transient increase in propidium iodide staining, a dye that stains charged polymers in the CW⁵³,
310 and high amplitude $[\text{Ca}^{2+}]_{\text{cyt}}$ and pH_{ext} transients that coincided with rapid tip swelling (Fig. S6A;
311 movie 6). No such changes were observed upon mock treatment (Fig. S6B, movie 6). Together
312 these observations underscore the dynamic nature of pectin metabolism in growing RHs, where
313 rapid feed-back signaling compensates the effect of the inhibition of PME activity, at least in part
314 through the alkalinization of the cell surface. The latter is expected to promote PME activity,
315 given the high pH optimum of plant PMEs³⁴. The radial expansion of the RH tip as rapid as one
316 minute after EGCG treatment emphasizes the critical role for pectin demethylesterification in
317 maintaining polar growth of RHs.

318 Next, we hypothesized that preventing pectin demethylesterification in the RH CW would also affect
319 the cell's response to RALF22 treatment. To test this, we measured $[\text{Ca}^{2+}]_{\text{cyt}}$ responses upon RALF22
320 treatment, in the presence or absence of EGCG, in seedlings expressing the G5A BRET sensor driven
321 by the RH-specific promoter pEXPa7⁵⁴. This assay, in contrast to the observation of individual RHs in
322 microfluidic chips (Figure 2; movie 2), is more suitable for gathering quantitative data from large
323 numbers of treatments. We found that a treatment as short as 30 min, at an EGCG concentration
324 close to the EC₅₀ for root growth inhibition ($32 \mu\text{M}$ ⁵⁵), greatly reduced the subsequent RALF22-
325 induced $[\text{Ca}^{2+}]_{\text{cyt}}$ transient (Fig. S6C, D), showing that, also *in vivo*, pectin demethylesterification is
326 crucial for RALF22 function.

327

328 To further investigate the *in vivo* connection between the methylesterification status of HG and
329 the periodic organization of (LRX1-)RALF22 in the CW, we labeled *ralf22-2 x pRALF22::mCherry-*
330 *RALF22_{mature}* RHs with monoclonal antibodies (mABs) directed against different HG epitopes (Fig.
331 6, Fig. S7). LM20, which labels methylesterified HG⁵⁶, was enriched at the tip and labeled sparse
332 patches along the RH shank (Fig. S7A-C), corroborating that methylesterified HG is deposited and
333 demethylesterified in the expanding dome. Interestingly, LM20 labeled an outer layer of the CW
334 that was devoid of mCHERRY-RALF22_{mature}, which localized closer to the plasma membrane both

335 in the tip and the shank (Fig. S7A, B). The mAb 2F4, an IgG1 that binds to Ca²⁺-crosslinked
336 demethylesterified HG⁵⁷, labeled the RH tip and shank and, like LM20, preferentially labeled an
337 outer layer of the CW, which only partially overlapped with mCherry-RALF22_{mature} (Fig. S7D, E;
338 Costes p-value>0.95; $tM_{mCherry-RALF22 \rightarrow 2F4} = 0.56 \pm 0.04$; $tM_{2F4 \rightarrow mCherry-RALF22} = 0.49 \pm 0.02$; n=8). Also
339 along the shank, Ca²⁺-crosslinked demethylesterified HG was organized in rings, where regions
340 with a relatively lower mCherry-RALF22_{mature} abundance appeared to show higher 2F4 labeling
341 and vice versa (Fig. S7E, F). Finally, PAM1, a small HIS-tagged scFv mAb directed towards long
342 (>30DP) stretches of block-wise demethylesterified HG⁵⁸, labeled the RH CW both in the tip and
343 shank (Fig. 6A-D). In contrast to LM20 and 2F4 epitopes, this epitope revealed circumferential
344 rings in the RH CW that largely overlapped with mCherry-RALF22_{mature} (Fig. 6A-D; Costes p-
345 value>0.95; $tM_{mCherry-RALF22 \rightarrow PAM1} = 0.74 \pm 0.05$; $tM_{PAM1 \rightarrow mCherry-RALF22} = 0.74 \pm 0.04$; n=12). These results,
346 in accordance with the charge-dependence of the (LRX1-)RALF22-pectin interaction (Fig. 3B, D-G,
347 Fig. 5I-K, Fig. S6C, D), show that newly secreted pectin, upon demethylesterification, interacts
348 with (LRX1-)RALF22 *in muro* where it becomes part of periodic rings (Fig. 6A-D).
349 The periodic CW pattern could indicate that mCherry-RALF22_{mature} associates with a preexisting
350 HG pattern in the CW or that the (LRX1-)RALF22 interaction with demethylesterified HG could be
351 responsible for inducing the formation of periodic pectin rings. To investigate this possibility, we
352 labeled the CW of *ralf22-2* RHs with anti-HG antibodies (Fig. 6E, F). Interestingly, in *ralf22-2*, the
353 abundance of block-wise demethylesterified HG (PAM1) and Ca²⁺-crosslinked demethylesterified
354 HG (2F4) was reduced by 68±3% and 46±7% respectively, relative to Col-0 (Fig. 6E, F). In addition,
355 methylesterified HG (LM20), showed a 95±3% and 85±16% reduced labeling in the *ralf22-2* tip
356 and shank, respectively, as compared to Col-0 (Fig. 6E, F). Combined, these data illustrate that, in
357 the absence of RALF22, overall pectin secretion is reduced and/or turnover is enhanced.
358 We then investigated the requirement of LRX1/2 for the periodic organization of the pectic CW
359 by studying the RHs of the double *lrx1-1/2-1* mutant expressing mCherry-RALF22_{mature} (*lrx1-1/2-1*
360 *x pRALF22::mCherry-RALF22_{mature}*, Fig. 6G-I, movie 7). These RHs, like those of the untransformed
361 *lrx1-1/2-1* parent, grew slowly and frequently burst. In surviving *lrx1-1/2-1* RHs, most mCherry-
362 RALF22_{mature} accumulated in intracellular compartments, yet a small amount was still secreted to
363 the CW (Fig. 6G, movie 7). CW labeling was 3.5- and 2.5-fold weaker in the tip and shank CW
364 respectively, compared to the complemented *ralf22-2 x pRALF22::mCherry-RALF22_{mature}* line (Fig.
365 6G, H, movie 7). Interestingly, in the absence of LRX1 and LRX2, mCherry-RALF22_{mature} and PAM1
366 organization showed a much more fragmented pattern compared to the control (Fig. 6D, I).
367 Moreover, in the *lrx1-1/2-1* CW, demethylesterified HG accumulated 2.4-fold, relative to the WT,
368 at the tip, but not in the shank (Fig. 6G, H). Given that pectin secretion occurs at the tip, this
369 suggests an increased pectin secretion and turnover in the absence of LRX1/2. Moreover, the
370 PAM1 epitope and mCherry-RALF22_{mature} showed only limited colocalization in the shank of *lrx1-1/2-1*
371 RHs (Fig. 6I; Costes p-value>0.95 for 4 out of 6 samples; $tM_{mCherry-RALF22 \rightarrow PAM1} = 0.25 \pm 0.08$;
372 $tM_{PAM1 \rightarrow mCherry-RALF22} = 0.19 \pm 0.07$; n=6). Together, these results show that the LRX1/2-RALF22
373 complex, through its binding and compaction of demethylesterified pectin, organizes the
374 patterning of the pectic RH CW. In the absence of this patterning, pectin secretion and turnover
375 are increased and the CW's integrity is compromised.

376 Discussion

377 Here, we identified RALF22 as the main trichoblast-expressed RALF peptide controlling growth
378 and CW integrity of RHs. RALF22 binds LRX1 and LRX2, forming extremely stable complexes
379 (dissociation $t^\circ > 80^\circ\text{C}$) *in vitro* (Fig. 5D-F) and, given the colocalization pattern (Fig. 5G-H), most
380 likely also *in vivo*. RALF22 also binds LLG1 and recruits FER into an active membrane-bound
381 ternary signaling complex (Fig. 2B, C). The *in vivo* relevance of this interaction in RHs is shown by

382 the requirement of FER to induce sustained surface alkalization and growth arrest upon
383 RALF22 treatment (Fig. 2E-J, movie 2).

384 What is the functional relevance of the existence of two distinct, mutually exclusive binding
385 partners²⁹ for the same peptide at the surface of the same cell? This report provides an answer
386 to this key question by showing that RALF22 is not only a signaling peptide, but that it also has a
387 structural role (Fig. 7). Indeed RALF22, as a free peptide or bound to LRX1, binds
388 demethylesterified pectin *in vitro* (Fig. 3B-G, Fig. 5I-K). Pectin binding is also relevant *in vivo*, as
389 suggested by (i) the diminished $[Ca^{2+}]_{cyt}$ response upon RALF22 treatment of RHs pretreated with
390 EGCG (Fig. S6C, D), (ii) the colocalization of mCHERRY-RALF22 with blockwise demethylesterified
391 HG (PAM1 epitope; Fig. 6A-D), but not with highly methylesterified HG (LM20 epitope; Fig. S7A-
392 C), in the RH CW, where it forms stable microdomains organized in periodic concentric rings (Fig.
393 4C-I, Fig. S5) and (iii) the loss of proper pectin organization in the absence of RALF22 or LRX1/2
394 (Fig. 6E-I).

395 A mechanism for microdomain formation is suggested by the capacity of free RALF22 and
396 LRX1_{LRR}-RALF22 to induce the compaction of a pectin layer. The charge-dependence of this
397 interaction suggests that the free unstructured and positively-charged peptide induces water
398 loss through the neutralization of the pectin charges and the resulting loss of osmotically active
399 counterions in the pectin gel. The interaction of the LRX1_{LRR}-RALF22 hetero-tetramer with pectin
400 may be more complex, since here the peptide is folded and exposes a structured surface with
401 positively charged residues that, besides electrostatic interactions, may also recognize structural
402 features of the polysaccharide, such as charge patterns, as reported for instance for GAG-binding
403 proteins in the animal ECM^{1,59}, or acetyl⁶⁰ or xylosyl⁶¹ substitution motifs. In addition, the
404 LRX1_{LRR}-RALF22 dimer may promote polymer crosslinking, thus reducing the water binding
405 capacity (Fig. 3D-G, Fig. 5I-K, Fig. S4A, B)⁶². Compaction of GAGs by GAG cross-linking proteins
406 creates denser zones in the gel, but also augments the porosity of the non-compacted zones as
407 observed during the formation of perineural nets, the porous aggregated matrices surrounding
408 neurons¹⁶. A similar process may explain why RALF22 supplementation triggers the migration of
409 dextran-coupled FITC and TRITC to the RH CW (Fig. 2E, F, J, movie 2). It should be noted that,
410 besides the RALF22-binding LRR domain, LRX1 also comprises a C-terminal extensin domain. At
411 least one extensin family member, AtEXT3, was able to polymerize into a scaffold *in vitro* thanks
412 to its amphiphilic properties⁶³. The authors hypothesized that this scaffold could form
413 coacervates with pectin, due to its repeated basic domains, and thus structure the CW. This
414 polymer network could then be consolidated by intermolecular oxidative crosslinking of the
415 extensin's tyrosine residues. Interestingly, since the extensin domain of LRX1 itself does not have
416 such basic domains, RALF22 may play this pectin-recruiting role in the complex. The advantage
417 of such a modularity might be twofold: first the same LRX could bind different CW
418 polysaccharides or polysaccharide motifs by switching RALF isoforms, perhaps promoting
419 different CW architectures, and second, free RALF peptide may be part of a FER-dependent
420 feedback signaling mechanism that coordinates its own incorporation into the CW.

421 This study provided striking new insights into the logic of RALF feedback signaling. Whereas
422 RALF22 treatment resulted in a dramatic change in the physicochemical properties of the CW,
423 the cell required FER to induce a normal pH and growth response (Fig. 2E-J, Fig. S3B-E, movie 2).
424 One way to explain these results is by assuming that the LLG1-RALF22-FER complex acts as a
425 mechano-sensor, which responds to CW compaction induced by (LRX1-)RALF22. In other words,
426 (LRX1-)RALF22 at the same time creates a signal (CW compaction) and controls the sensitivity of
427 the detection system, possibly through LLG1-RALF22-FER recruitment. A role for LLG1/FER in
428 mechano-sensing has been proposed previously⁶⁴⁻⁶⁶. For instance, stretched epidermal cells

429 upon root bending displayed biphasic cytosolic Ca^{2+} responses, with a rapid transient and a
430 slower more sustained component. Only the latter disappeared in *llg1* and *fer* mutants⁶⁵,
431 whereas the initial transient might come from another mechanosensor, such as a
432 mechanosensitive Ca^{2+} -permeable channel⁶⁷. Similarly in RHs, we observed that in *fer-4*, RALF22
433 still induced a transient growth inhibition and surface pH increase at the tip, after which growth,
434 pH and $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations resumed, in contrast to the wild type, where the surface
435 alkalisation propagated along the shank and growth did not recover (Fig. 2E-J, Fig. S3B-E,
436 movie 2). Mechanosensing in the leaf epidermis also appears to depend on FER, where it signals
437 through the activation of ROP6 by FER-bound ROPGEF14⁶⁵. This in turn is thought to affect
438 microtubule and actin dynamics and secretion. Interestingly, we observed an over 90% reduction
439 in methylesterified (LM20) pectin at the RH tip of *ralf22* and an over two-fold increase of the
440 epitope in *lrx1/2* (Fig. 6H, I). This suggests that free RALF22, which is absent in *ralf22* and
441 expected to be more abundant in the absence of LRX1/2, may promote the secretion of pectin at
442 the tip.

443 RALF22-induced and FER-dependent sustained alkalization is also expected to promote HG
444 demethylesterification given the alkaline pH optimum of PMEs³⁴. Together, free RALF22 might
445 signal the need for more polyanionic pectin as a substrate for the CW-structuring LRX1-RALF22-
446 pectin complexes. In this context it is of interest to note that the spacing of the CW
447 microdomains in the RH tip matched the length increase during a growth/surface pH oscillation
448 cycle (Fig. 4I, Fig. S5), and that apical RALF22 deposition oscillated in antiphase with the growth
449 rate (Fig. 4G). This supports the idea that CW assembly is a cyclic process in RHs along the
450 following scenario (Fig. 7): first, wall material, including methylesterified HG and LRX1-bound
451 and free RALF22, is deposited at the tip of the growing RH. A concomitant or subsequent
452 increase in CW pH through the binding of free RALF22 to LLG1/FER, activates processive pectin
453 methylesterase (PME) activity, thus exposing stretches of negative charges on the polymer. This
454 creates the substrate for the interaction with the LRX1/2-RALF22 dimer causing the dewatering
455 of the pectin gel and the formation of a ring-like structure of higher density surrounding the RH
456 tip. This structure is organized in such a way that it favors CW expansion in the circumferential
457 direction. This deposition-demethylesterification-compactation-expansion process is repeated
458 during each growth cycle. Given that cellulose⁶⁸, xyloglucan⁶⁹ and expansins⁷⁰ are essential for
459 RH growth, it will be interesting to see if expansin-mediated acid growth contributes to the
460 preferentially circumferential expansion of the ring and whether this involves purely remodeling
461 of the existing structure or the insertion of additional material. Further studies using microscopy
462 at high spatio-temporal resolution¹¹, should provide more insights into this fascinating CW
463 assembly process in RHs and most likely in other cell types in plants.

464 Acknowledgments

465 This work was funded by the Research Foundation Flanders (FWO; grants 1225120N and G013023N),
466 the University of Antwerp (UA; BOF-KP; DOCPRO4) and LASERLAB-EUROPE (grant 654148) to SS
467 and KV; the Agence Nationale pour la Recherche (ANR), project "HOMEOWALL" to HH; and the
468 University of Lausanne, the European Research Council (ERC) grant agreement no. 716358 and the
469 Swiss National Science Foundation grant 310030_204526 to JS. The authors would like to thank Jozef
470 Mravec for sharing OG7-13, and Christoph Ringli, Alice Cheung and Simon Gilroy for kindly donating
471 *lrx1-1/2-1* and *lrx1-1 x pLRX1::cmcy-LRX1*, *fer-4 x pFER::FER-GFP*, and Col-0 x 35S::GCaMP3 seeds,
472 respectively. Tou Cheu Xiong is thanked for providing the G5A Ca^{2+} sensor line. Our gratitude also
473 goes to CPKelco (Denmark) for donating industrial low and high methoxyl pectin samples. The IJPB
474 benefits from the support of Saclay Plant Sciences-SPS (ANR-17-EUR-0007). This work has benefited
475 from the support of IJPB's Plant Observatory technological platforms. Access to fluorescence

476 microscopy infrastructure was provided by the Antwerp Centre for Advanced Microscopy (ACAM,
477 UA). The purchase of the PerkinElmer UltraVIEW Vox and Leica SP8 confocal microscopes was
478 supported by FWO-HERCULES infrastructure grants; AUHA-09-001, AUHA-15-12. The Nikon Sora
479 microscope was purchased with the support of an FWO mid-size infrastructure (I003420N) and FWO
480 IRI grant (I000123N).

481 **Author contributions**

482 S.S., K.V., H.H. and J.S. conceived the project. S.M. aided in conceptualizing the research based on his
483 work on LRX8-RALF4. J.K.L. and J.S. designed, produced and characterized all recombinant proteins.
484 C.B. provided technical assistance for protein production. E.F. and M.G. designed and performed
485 calcium assays using the G5A BRET sensor. S.S. generated all crosses and performed the cloning of
486 the pEXPa7::G5A sensor and RALF22-related constructs. S.S. optimized microfluidics for imaging and
487 performed the phenotyping, staining and imaging of live cell and fixed-tissue samples. S.S. performed
488 image analysis, bio-informatics and statistics. N.C. assisted with live cell imaging, cloning and sample
489 preparations. D.B. assisted with cloning, phenotyping and sample preparation. S.S. and M.G.
490 performed Microscale Thermophoresis analysis. H.H. and B.C. conceived the QCM-D analysis. T.L. and
491 H.H. performed the QCM-D analysis. T.L. characterized the pectin solutions. E.B. and C.M. provided
492 technical and conceptual assistance with the *in vitro* QCM-D and pectin work. H.A. aided in
493 conceptualizing the work and provided assistance with sample preparation for phenotyping. A.B.,
494 A.C., D.S.C.D and J.F. aided in the visualization and analysis of oscillatory parameters in live cell
495 imaging. J.S. generated 3D protein structures and performed peptide docking modelling. S.S., H.H.
496 and K.V. wrote the manuscript.

497 **Declaration of interests**

498 The authors declare no competing interests.

499 **Figure legends**

500 Figure 1. RALF22 regulates root hair growth. (A) scheme of the *RALF22* genomic sequence showing
501 the T-DNA insertion site of GabiKat line GK_293H03 (*ralf22-2*) and the 182 nucleotide-long deletion
502 in *ralf22-1*. (B) representative 6-day-old roots of Col-0, *ralf22-1* (-/-), *ralf22-2* (-/-) and *ralf22-2* x
503 *pRALF22::RALF22* (COMP) seedlings (scale bar=500µm). Close-ups of RHs showing the short, bulged
504 and burst (cfr. movie 1) *RALF22* loss-of-function phenotype (scale bar=100µm). (C) graph showing the
505 mean RH length (±SEM) and % of RH bursting for all genotypes (n≥5, ***p<0.001). Three
506 independent complementation lines (*ralf22-2* x *pRALF22::RALF22*) are shown. (D) representative
507 confocal maximal projections and transverse optical sections of 6-day-old Col-0 seedlings expressing
508 GFP under the control of the *RALF22* promoter (Col-0 x *pRALF22::GFP*). *RALF22* is transcribed in
509 trichoblast cells throughout RH growth (scale bars= left, 100µm; middle, 50µm; right; 25µm).

510

511 Figure 2. RALF22 binds to FERONIA to regulate root hair growth. (A) model of mature RALF22
512 showing the conserved YISY domain (sticks) required for LLG binding. (B) MST results showing the
513 binding affinity between fluorescently labelled recombinant LLG1 and RALF22 (n=7) or RALF22^{Y75A,Y78A}
514 (n=3). (C) Binding affinity between the FERONIA ectodomain (FER_{ecd}) and fluorescently labelled LLG1
515 preincubated with RALF22 (n=3) or RALF22^{Y75A,Y78A} (n=3). MST values are represented as the average
516 normalized change in fluorescence (%) ± SEM. (D) Homology model of the LLG2-RALF22-FER_{ecd}
517 complex. The N-terminal region of RALF22 was docked into the LLG2 structure (PDB:6A5E). FER_{ecd} was
518 superimposed based on the crystal structure. The YISY motif is highlighted in sticks. (E-F) Timelapse
519 imaging of Col-0 (E) and *fer-4* (F) RHs being treated with 5µM RALF22 (cfr. Movie 2). The kymographs
520 and corresponding plots depict changes in the growth rate, [Ca²⁺]_{cyt} (cytosolic GCaMP3 fluorescence
521 intensity), pH_{ext} (extracellular FITC-110kDa dextran/TRITC-20kDa dextran fluorescence intensity ratio)

522 and migration of fluorescent FITC-110kDa dextran and TRITC-20kDa dextran into the CW (a proxy for
523 altered CW physico-chemistry) during 4min of steady state growth followed by treatment with
524 RALF22. The RH's response to RALF22 was monitored for 6min. Kymographs were generated along
525 the horizontal lines depicted in the insets. (G-J) quantification of the average growth rate (G), $[Ca^{2+}]_{cyt}$
526 (H), pH_{ext} (I), and FITC or TRITC fluorescence in the CW (J) before, upon and after treatment with
527 RALF22 in Col-0 (n=9) and *fer-4* (n=8) RHs. Col-0 RHs were also treated with a mock solution (-
528 RALF22, n=7). Barplots represent the mean \pm SEM. Different letters represent statistical significance
529 ($\alpha=0.05$).

530

531 Figure 3. RALF22 binds and compacts demethylesterified homogalacturonan. (A) mature RALF22 is a
532 positively charged protein. In bold: the amino acids which are positively charged at pH 7.0. (B) MST
533 affinity plots showing that RALF22 interacts with OG7-13, a 7-13mer long fully demethylesterified
534 oligogalacturonide (n=3). Mutating three cationic arginines on the RALF22 (RALF22^{R82A, R90A, R100A})
535 surface leads to a 3.7-fold reduction in affinity (n=3). (C) illustration of the QCM-D working principle.
536 An alternating current is applied to a quartz surface which holds the pectin gel. The frequency of the
537 lateral quartz oscillation (F) is inversely correlated with the mass of the pectin layer. The time it takes
538 for the lateral oscillation to dissipate (D) when the current is interrupted positively correlates with
539 the rigidity of pectin layer. (D-F) Representative ΔF and ΔD QCM-D traces for a pectin layer with a
540 degree of methylesterification of 75% (D) or 31% (E) treated with $1\mu g \cdot mL^{-1}$ RALF22 or DM31 pectin
541 treated with $1\mu g \cdot mL^{-1}$ RALF22^{R82A, R90A, R100A}. Grey zones highlight the intervals during which pectin or
542 RALF were applied (cfr. Fig. S4A,B). (G) Quantification of the % change in ΔF and ΔD upon RALF
543 treatment for the conditions presented in D-F (DM75 + RALF22, n=14; DM31 + RALF22, n=14; DM31 +
544 RALF22^{R82A, R90A, R100A}, n=2). Data is shown as the mean \pm SEM. Different letters represent statistical
545 significance ($\alpha=0.05$).

546

547 Figure 4. RALF22 forms circumferential rings in the root hair cell wall which illustrate anisotropic wall
548 expansion. (A) representative images of 6-day-old Col-0, *ralf22-2* and *ralf22-2 x pRALF22::mCHERRY-*
549 *RALF22_{mature}* roots (scale bar=500 μm) and RHs (scale bar=100 μm). (B) quantification of the average
550 RH length and percentage of RH bursting for each genotype (n \geq 5, ***p<0.001). (C) Representative
551 longitudinal optical section of a growing (left) and a mature (right) *ralf22-2 x pRALF22::mCHERRY-*
552 *RALF22_{mature}* RH (scale bar=8 μm). ROIs showing the presence of mCHERRY-RALF22_{mature} microdomains
553 in the RH CW (scale bar=2 μm). (D) maximal projection kymograph corresponding to a 10min
554 timelapse acquisition of a growing *ralf22-2 x pRALF22::mCHERRY-RALF22_{mature}* RH. Vertical red lines
555 represent mCHERRY-RALF22_{mature} microdomains that remained stationary in the CW throughout the
556 acquisition. The longitudinal fluorescence pattern for quantification of the mCHERRY-RALF22_{mature}
557 microdomain spacing was extracted along the yellow dotted line. (E) maximal projection kymograph
558 of the RH tip CW after removal of the dome's concave shape. Red lines represent stationary
559 mCHERRY-RALF22_{mature} microdomains, which arise at the very apex and move towards the shank as
560 the tip grows forward. (F) Representative snapshots of a growing *ralf22-2 x pRALF22::mCHERRY-*
561 *RALF22_{mature}* RH before, upon and after photobleaching of mCHERRY-RALF22_{mature} in ROIs in the CW of
562 the expanding dome, below the tip and in the shank (scale bar=5 μm). Graphs showing mCHERRY
563 fluorescence recovery at the apex only. (G) representative timeseries showing that RH growth rate
564 and apical mCHERRY-RALF22_{mature} fluorescence oscillate in antiphase. (H) frontal and lateral high-
565 resolution z-projections of mCHERRY-RALF22_{mature} organization in the RH CW, illustrating the
566 formation of regularly spaced mCHERRY-RALF22_{mature} rings (scale bar=2 μm). (I) Representative high
567 resolution longitudinal optical section of a representative growing RH (scale bar=5 μm) showing
568 mCHERRY-RALF22_{mature} microdomains in the tip and the shank (scale bar=4 μm). Comparative analysis
569 of mCHERRY-RALF22_{mature} microdomain spacing in the tip (n=12) vs. the shank (n=15). Data is
570 presented as the mean \pm SEM. Asterisks depict statistical significance (**p<0.01).

571

572 Figure 5. RALF22 binds to LRX1 in the root hair cell wall and compacts demethylesterified
573 homogalacturonan. (A) The *lrx1-1/2-1* and *ralf22-2* phenotypes are indistinguishable. Representative

574 pictures (scale bar=500 μ m) and close-ups (scale bar=100 μ m) of 6-day-old seedlings are shown. (B)
575 quantification of the RH length and % of RH bursting of Col-0, *ralf22-2* and *lrx1-1/2-1* seedlings (n \geq 5,
576 ***p<0.001). (C) Structural superimposition of the LRX2 (PDB:6QXP; grey) and LRX1 AlphaFold2
577 model (orange) (top left). Root Mean Square Deviation (R.M.S.D) of 0.15 \AA comparing 342 pairs of
578 corresponding C α atoms between the two proteins. Full molecular docking model of RALF22 (green)
579 with LRX2_{LRR} (PDB: 6QXP; grey) (top right). Enlarged view of the peptide docking models showing
580 RALF22 (green) in the LRX2_{LRR} and LRX1_{LRR} (AlphaFold2 model) binding pockets (bottom). The three
581 exposed Arginines (R82, R90, R100) required for pectin binding are depicted as sticks. (D) Size
582 exclusion chromatography of LRX1_{LRR}-RALF22 (pH 5.0 and 2.0) and LRX2_{LRR}-RALF22 (pH 5.0) purified
583 from insect cells, showing that the complex does not dissociate at pH 2. (E) SDS protein gels of the
584 fractions corresponding to the SEC elution peaks for LRX1_{LRR}-RALF22 (pH 5.0 and 2.0) and LRX2_{LRR}-
585 RALF22 (pH 5.0). (F) thermoshift assay of LRX8_{LRR}-RALF4 (control), LRX1_{LRR}-RALF22 and LRX2_{LRR}-
586 RALF22. LRX1_{LRR}-RALF22 was subjected to pH 5.0 and pH 2.0. Low pH does not affect the
587 thermostability of the complex. (G) representative longitudinal optical sections of the *lrx1-1* x
588 *pLRX1::cMyc-LRX1* x *pRALF22::mCHERRY-RALF22_{mature}* CW in which LRX1 was labeled with a green
589 fluorescent anti-cMyc antibody (scale bar=5 μ m). Dots represent cMyc-LRX1 (green) and mCHERRY-
590 RALF22_{mature} microdomains (red). Composite images and magnifications (scale bar=1 μ m) show
591 colocalization (yellow) between LRX1 and RALF22. (H) Representative lateral z-projections of cMyc-
592 LRX1 (green) and mCHERRY-RALF22_{mature} (red) organization and colocalization (yellow) in the RH CW
593 (scale bar=5 μ m). cMyc-LRX1 colocalizes with RALF22_{mature} rings in the CW. (I,J) representative Δ F and
594 Δ D traces of DM75 (I) and DM31 (J) treated with 10 μ g.mL⁻¹ of LRX1_{LRR}-RALF22. (K) Quantification of
595 the % change in Δ F and Δ D (relative to the pectin-induced change) upon LRX1_{LRR}-RALF22 treatment
596 for the conditions presented in I and J (DM31 + LRX1_{LRR}-RALF22, n=11; DM75 + LRX1_{LRR}-RALF22, n=6).
597 Data is shown as the mean \pm SEM. Asterisks represent statistical significance (p-value; ***<0.001).
598

599 Figure 6. (LRX1-)-RALF22-homogalacturonan interaction regulates the organization of the pectic root
600 hair cell wall. (A-D) representative images showing the colocalization of PAM1 (>30DP long stretches
601 of block-wise demethylesterified homogalacturonan) and mCHERRY-RALF22_{mature} in the RH tip and
602 shank. (A) longitudinal optical sections of a PAM1 (green) and mCHERRY-RALF22_{mature} labeled RH
603 (scale bar=10 μ m), which was fixed while growing. Yellow indicates colocalization in the composite
604 image. (B) close-ups of RH tip presented in A showing that PAM1 and mCHERRY-RALF22_{mature}
605 colocalize along the growing dome (scale bar=2.5 μ m). (C) representative longitudinal optical sections
606 of the shank CW and ROIs showing the presence of overlapping microdomains of demethylesterified
607 HG (PAM1; green) and mCHERRY-RALF22_{mature} (red) (scale bar=1 μ m). (D) lateral z-projections of the
608 RH shank CW showing the formation of colocalized PAM1 and mCHERRY-RALF22_{mature} circumferential
609 rings (scale bar=5 μ m). (E) representative longitudinal optical sections of *ralf22-2* RHs labeled with
610 PAM1 (demethylesterified HG), 2F4 (Ca²⁺-crosslinked demethylesterified HG) and LM20
611 (methylesterified HG) (scale bar=10 μ m). Asterisks indicate expelled intracellular debris as a result of
612 cell bursting. (F) quantification of the labeling intensities for the PAM1 (n_{col-0}=12; n_{PAM1}=16), 2F4 (n_{col-0}=
613 8; n_{2F4}=9) and LM20 epitopes (n_{col-0}=19; n_{PAM1}=9) relative to Col-0 (top barplot), and the ratio
614 between between tip and shank methylesterified HG abundance (LM20; lower barplot). Data is
615 shown as the mean \pm SEM. Asterisks represent statistical significance (p-value; **<0.01; ***<0.001).
616 (G) representative longitudinal optical sections of the tip and shank CW of a surviving PAM1 (green)-
617 labelled *lrx1-1/2-1* RH expressing mCHERRY-RALF22_{mature} (red). Yellow indicates colocalization in the
618 composite frame (scale bar=5 μ m). (H) Quantification of the PAM1 (green) and mCHERRY-RALF22_{mature}
619 (red) CW labeling intensities in the tip and shank of *lrx1-1/2-1* x *pRALF22::mCHERRY-RALF22_{mature}* and
620 complemented *ralf22-2* x *pRALF22::mCHERRY-RALF22_{mature}* RHs (left half) alongside the tip/shank
621 labeling ratio (right half). Data is shown as the mean \pm SEM. Different letters and asterisks represent
622 statistical significance (p-value; ***<0.001). (I) lateral z-projections of the PAM1 labeled *lrx1-1/2-1* x
623 *pRALF22::mCHERRY-RALF22_{mature}* RH shank CW showing the fragmented pattern of
624 demethylesterified HG and RALF22 organization (scale bar=5 μ m).
625

626 Figure 7. Model of the dual structural and signaling role of RALF22 in the periodic assembly of the
627 root hair cell wall. (A) RALF22, LRX1-RALF22 and methylesterified HG are secreted at the growing RH
628 tip. RALF22 forms a ternary complex with LLG1 and FER, thereby regulating its own secretion and
629 inducing downstream cytosolic calcium signaling and alkalization of the apical CW. In the CW,
630 Pectin Methyl Esterases (PMEs), which have a high pH optimum, catalyze HG demethylesterification,
631 generating anionic HG through the formation of stretches of negatively charged carboxyl groups.
632 RALF22 and LRX1-RALF22, electrostatically interact with poly-anionic demethylesterified HG. This
633 induces HG dewatering and compaction, and the self-assembly of the HG matrix into (LRX1-)RALF22-
634 HG microdomains. (B) These microdomains represent periodic circumferential (LRX1-)RALF22-HG
635 rings which originate in the growing tip, and arise as a consequence of oscillatory (LRX1-)RALF22
636 secretion at the tip and apical pH oscillations which catalyze periodic HG demethylesterification. This
637 temporal periodicity is translated towards the spatial periodicity of (LRX1-)RALF22-HG ring assembly.
638 (LRX1-)RALF22-HG rings illustrate highly anisotropic CW expansion, in which the circumferential
639 strain of the rings greatly surpasses the meridional strain, which is a prerequisite for polar RH
640 growth.
641

642 **Methods**

643 **Plant materials**

644 *Arabidopsis thaliana* ecotype Columbia-0 (Col-0; N1092), *ralf22-2* (GK_293H09; N428125;
645 At3g05490), *fer-4* (GK_106A06; N69044; At3g51550), *eru* (SALK_083442; N583442; At5g61350),
646 and *llg1-2* (SALK_086036; N586036; At5g56170) seeds were obtained from the Eurasian
647 Arabidopsis Stock Centre (uNASc). Genotyping of *ralf22-2* plants was performed by PCR using T-
648 DNA (GTAGATTTCCCGACATGAAGCCA) and gene-specific primers (FW:
649 ACCGGTCAACCAGTTTCTGCAT, REV: TTCAACGCCTGCACCTAGTGAT). The *ralf22-1* line was
650 generated using CRISPR-cas9. *Lrx1-1/2-1* (At1g12040, At1g62440) and *lrx1-1 x pLRX1::myc-LRX1*
651 seeds were kindly donated by Prof. Christoph Ringli. *Fer-4 x pFER::FER-GFP* was kindly donated
652 by Alice Chueng. Col-0 x 35S::GCaMP3 seeds were kindly provided by Prof. Simon Gilroy. *ralf22-1*,
653 Col-0 x *pEXPa7::G5A*, *ralf22-2 x pRALF22::mCHERRY-RALF22_{mature}*, *lrx1-1/2-1 x*
654 *pRALF22::mCHERRY-RALF22_{mature}* and *lrx1-1 x pLRX1::myc-LRX1/pRALF22::mCHERRY-*
655 *RALF22_{mature}* lines were generated during this study. *Eru x pERU::ERU-GFP* was generated
656 previously⁴².

657 **Growth conditions**

658 Seeds were surface sterilized and sown on RH growth medium (3mM KNO₃, 2mM
659 Ca(NO₃)₂·4H₂O, 0.5mM MgSO₄·7H₂O, 1mM NH₄H₂PO₄, 1mg.mL⁻¹ thiamine, 0.5 mg.mL⁻¹
660 pyridoxine-HCl, 0.5mg.mL⁻¹ nicotinic acid, 0.56mM myo-inositol, 25mM KCl, 17.5mM H₃BO₃,
661 1mM MnSO₄·H₂O, 1mM ZnSO₄·7H₂O, 0.25mM CuSO₄·5H₂O, 0.25mM (NH₄)₆MoO₂₄·4H₂O, 25mM
662 Fe-Na EDTA, 0.8% gelrite or phytagel, 1% sucrose, 2.3mM MES at pH 5.7) for phenotyping or
663 basal Murashige and Skoog (MS) medium (1% sucrose, 1% plant agar, 0.5g.L⁻¹ MES at pH 5.8)
664 prior to microfluidic preparation. For live cell imaging and immunolocalization assays, plants
665 were transferred to microfluidic chips and grown overnight in liquid medium (0.1mM KCl,
666 0.1mM CaCl₂, 1mM NaCl, 1% sucrose, 0.5g.L⁻¹ MES at pH 6.0). Imaging of extracellular pH
667 oscillations was performed in unbuffered liquid medium at pH 6.0.

668 Seeds were stratified for 2-3 days in the dark at 4°C and plates or microfluidics chips were placed
669 vertically in a growth chamber with standard growth conditions (16h light-8h dark, 22°C).

670

671 **Identification of RH-specific RALFs**

672 Using the BAR ePlant Browser⁷¹, Root-specific transcription was assessed for all *RALFs* that are
673 represented on Affymetrix ATH1 arrays. Root-expressed, trichoblast-specific *RALFs* were
674 identified from single cell RNAseq data based on expression in cluster 15 (trichoblasts) in T-SNE
675 plots³⁶. For *RALF22*, a trichoblast pseudotime expression profile was generated, showing *RALF22*
676 expression in function of the trichoblast's distance to the meristem³⁷.

677 Immunolocalization

678 Four day old Col-0, *ralf22-2*, *ralf22-2 x pRALF22::mCHERRY-RALF22_{mature}*, *lrx1-1 x pLRX1::cmyc-*
679 *LRX1/pRALF22::mCHERRY-RALF22_{mature}* and *lrx1-1/2-1 x pRALF22::mCHERRY-RALF22_{mature}*
680 seedlings were grown overnight in microfluidics chips⁷² and fixed for 10min in buffered liquid
681 medium containing 10% acetic acid and 3.7% formaldehyde. All subsequent steps were
682 performed in buffered liquid medium. Each channel was washed (3x5min) and incubated for
683 15min with 50mM NH₄Cl to neutralize residual formaldehyde. After washing (3x5min), aspecific
684 binding sites were blocked for 30min in 3% BSA and incubated overnight at 4°C in the presence
685 of 3% BSA and a 20-fold (2F4, LM20) or 40-fold (PAM1) primary antibody dilution. Seedlings
686 were washed (3% BSA, 3x5min) and incubated for 30min at RT with a 50-fold secondary antibody
687 dilution (2F4; goat anti-mouse IgG-FITC, LM20; goat anti-rat IgM-Alexa⁴⁸⁸, PAM1; Rabbit anti-His-
688 DyLight⁴⁸⁸, cMyc-LRX1; anti-c-Myc-Alexa⁴⁸⁸). Following a final washing step (3x5min), the chips
689 with labeled seedlings were used for imaging on a Leica TCS SP8 confocal laser scanning
690 microscope (Leica-microsystems).

691 Microscopy

692 Six-day-old plants grown on RH growth medium were used for phenotyping. A Nikon AZ100
693 multizoom microscope or Zeiss AxioZoom v16 were used to collect 2h long timelapse
694 acquisitions or images of whole roots and individual RHs.

695 For fluorescence microscopy plants were grown overnight in uncoated IBIDI μ -slides VI 0.4 filled
696 with liquid medium⁷². Each channel contained a single plant.

697 Col-0 *x pRALF22::GFP* plants were counterstained by injecting 10 μ M propidium iodide (PI) (in
698 liquid medium) in each channel. Roots were imaged in xyz-mode using a Leica TCS SP8 confocal
699 laser scanning microscope (Leica-microsystems) equipped with a white light laser, 2 HyD
700 detectors and a 5x HC PL FLUOTAR dry objective (NA 0.15). GFP (excitation: 488nm, emission:
701 501-536nm and PI (excitation: 556, emission: 590-710nm) fluorescence were captured in
702 bidirectional line-scanning mode at 8-bit. The z-spacing was set to 1 μ m-2 μ m.

703 The same system with a 63x HC PL APO CS2 water immersion objective (NA 1.20) was used to
704 image [Ca²⁺]_{cyt}, pH_{ext}, growth rate and CW porosity or CW charge dynamics upon treatment with
705 *RALF22* or EGCG respectively. Col-0 and *fer-4* plants were grown in microfluidics chips in 220 μ L
706 of unbuffered liquid medium. Prior to imaging, 160 μ L of medium was removed from each
707 channel. 60 μ L of medium containing 0.29 mg.mL⁻¹ FITC-110kDa dextran and 0.25 mg.mL⁻¹ TRITC-
708 20kDa dextran (for *RALF22* treatment) or 0.29 mg.mL⁻¹ FITC-110kDa dextran and 10 μ M PI (for
709 EGCG treatment) was injected back into the channel. Plants were allowed to recover for 30min
710 and were subsequently imaged for 10-20min at 30 frames.min⁻¹. FITC/GCaMP3 (excitation:
711 488nm, emission: 501-548nm) and TRITC (excitation: 544nm, emission: 560-710nm) or PI
712 (excitation: 556nm, emission: 590-710nm) fluorescence were captured using bidirectional line-
713 by-line scanning in xyt-mode. After ~4min of steady state growth channels were injected with
714 60 μ L of liquid medium containing either 5 μ M *RALF22* or 100 μ M EGCG and imaged for another 6-
715 16min.

716 Timelapse acquisitions of *fer-4 x pFER::FER-GFP*, *eru x pERU::ERU-GFP*, *ralf22-2 x*
717 *pRALF22::mCHERRY-RALF22_{mature}* and *lrx1-1/2-1 x pRALF22::mCHERRY-RALF22_{mature}* were
718 acquired in buffered liquid medium on an inverted Nikon Ti wide-field microscope equipped with
719 a micro-lenses enhanced PerkinElmer UltraVIEW Vox spinning disk confocal system and a 60x

720 Plan Apo VC oil immersion objective (NA 1.40). GFP (excitation: 488 nm, emission: 525 nm) and
721 mCHERRY (excitation: 561 nm, emission: 615 nm) fluorescence were captured for 10-20 min at
722 30 frames.min⁻¹.

723 The photokinesis unit of the same system was used for Fluorescence Recovery After
724 Photobleaching (FRAP) experiments on *ralf22-2* x *pRALF22::mCHERRY-RALF22_{mature}* RHs. To this
725 end, growing RHs were imaged for 3-4min after which mCHERRY was bleached in 3 ROIs
726 corresponding to the growing tip, the shank just beneath the expanding dome and a region of
727 the shank ~100µm below the tip. Recovery of mCHERRY fluorescence was imaged for an
728 additional 10min.

729 High resolution 3D imaging of mCHERRY-RALF22_{mature} localization in the RH CW was performed
730 on a Nikon Ti2 W1-SoRa spinning disk system using the 561 nm laser line, 615 nm emission filter
731 and a 100x SR HP Plan Apo silicon immersion objective (NA 1.35). Images were collected in xyz
732 mode with a z-spacing of 0.25µm. 3D deconvolution was performed using NIS Elements AR,
733 based on a calculated Point Spread Function (PSF) and 10-20 iterations.

734 3D acquisitions of *ralf22-2* x *pRALF22::mCHERRY-RALF22_{mature}* labelled with LM20, 2F4 or PAM1
735 antibodies were collected in the same way using the 561 nm and 488 nm laser lines
736 respectively.

737 Cloning

738 The *ralf22-1* mutant was generated using CRISPR-cas9 mutagenesis to induce a 182-nucleotide
739 deletion downstream of the *RALF22* start codon. To this end, two domesticated guide RNA's
740 were simultaneously expressed (gRNA1: ATTGGCGATAGTAATCTCAGCCGGTTT; gRNA2:
741 ATTgTAGCTACGGTGCTATGAGGGTTT).

742 Other constructs were generated by synthesizing the fragment of interest into pDONR221
743 (ThermoFisher) and subsequent recombination to the desired destination vectors using gateway
744 cloning. The *pRALF22::GFP* transgene was constructed using the entire 2658bp *RALF22* promoter
745 sequence, including the 5'-UTR. To generate the *pRALF22::RALF22* construct, the entire *RALF22*
746 promoter and 360bp genomic sequence including STOP codon were synthesized. The
747 *pRALF22::mCHERRY-RALF22_{mature}* construct was generated by synthesizing the full *RALF22*
748 genomic sequence in which mCHERRY was inserted between the sequences coding for the
749 serine-protease cleavage site and the mature *RALF22* peptide. To generate a RH-transcribed G5A
750 [Ca²⁺]_{cyt} BRET sensor we synthesized a construct containing the 535bp promoter of the
751 *EXPANSINa7* gene and the G5A sequence⁵⁴. All pDONR221 plasmids were amplified in *E. coli*.
752 Positive colonies were identified by PCR using *pRALF22*- (FW: TTCTCTGACGCCGTCGACTTATC,
753 REV: GGGTAGCACTATTCTGCGTTGAC) and *GFP*-specific (FW: CACATGAAGCAGCAGACT, REV:
754 TGCTCAGGTAGTGGTTGTCG) primers. Transgenes were recombined into pFAST-R01 (no tag,
755 *pOLE1::RFP* seed marker), pFAST-R07 (C-terminal GFP, *pOLE1::RFP* seed marker) or pGWB1 (no
756 tag, Kan/hyg resistance) using the gateway LR reaction⁷³. *Agrobacterium tumefaciens* strain
757 LBA4404 transformed with the construct of interest was used to transform Col-0 or *ralf22-2*
758 plants using the floral dip method⁷⁴. To generate the *pRALF22::RALF22^{R82A,R90A,R100A}* construct, the
759 full *pRALF22* promoter and *RALF22* coding sequence (CDS) were amplified from *Arabidopsis*
760 *thaliana* Col-0 genomic DNA. Structure-based mutations were introduced in the *RALF22* CDS by
761 site-directed mutagenesis (R82A,R90A,R100A). The transgene was recombined into pGGZ003
762 (addgene ID:48869) using GreenGate assembly and subsequently transformed into
763 *Agrobacterium tumefaciens* strain ASE⁷⁵.

764 Image analysis

765 Image analysis was performed in Fiji. Root hair length was measured for 7 representative RHs
766 per root, and >20 roots grown on 5 plates.
767 For *pRALF22::GFP* stacks, the 3D viewer and z-project plugins were used to generate 3D
768 renderings and maximal projections of the acquired z-stacks.
769 To extract GCaMP3, FITC, TRITC and PI dynamics from timelapse acquisitions, individual frames
770 of the red channel were aligned at subpixel resolution using the template matching plugin and
771 normalized cross correlation as a matching method. The alignment coordinates were used to
772 calculate the subpixel displacement of each frame relative to the first frame, from which the
773 growth rate at each timepoint was calculated. The coordinates were then applied to the
774 corresponding green channel. $[Ca^{2+}]_{cyt}$ traces were extracted from circular ROI in cytoplasm at
775 the tip whereas the pH_{ext} was calculated from the FITC/TRITC ratio extracted from a ROI
776 positioned in the extracellular medium along the expanding dome. The pH_{ext} was calibrated by
777 imaging FITC and TRITC fluorescence in citric acid-sodium phosphate buffered liquid medium, at
778 the exact same imaging settings. A ROI encompassing the apical CW was defined to extract the
779 FITC and TRITC signal corresponding to a change in CW porosity or the PI signal corresponding to
780 changes in CW charge. To quantify changes in cell width, the lateral displacement of the PI-
781 stained CW just beneath the dome was calculated by laterally aligning the CW in a $1\mu m$ thin ROI
782 (from the center of the cell to the frame's edge). The alignment coordinates were used to
783 calculate the radial expansion of the cell upon EGCG treatment.
784 To visualize the mobility of mCHERRY-RALF22_{mature} microdomains, we generated kymographs for
785 a linear ROI overlaying the RH CW. To visualize microdomain mobility in the growing RH tip, a
786 kymograph was generated from the apical CW which was straightened in Fiji. The recovery of
787 mCHERRY-RALF22_{mature} fluorescence after bleaching was quantified by extracting the average
788 fluorescence intensity over time in a ROI overlaying the stretch of bleached shank CW. Tip
789 fluorescence recovery was quantified at the very apex after alignment of consecutive frames
790 using the template matching plugin.
791 To investigate the dynamics of RALF22 secretion, mCHERRY-RALF22_{mature} fluorescence was
792 quantified in a ROI at the very tip of frame aligned timelapse acquisitions, and correlated with
793 the RH growth rate oscillations that were calculated from the alignment coordinates.
794 RALF22 spacing in the CW was quantified by extracting the mCHERRY-RALF22_{mature} fluorescence
795 intensity pattern from the line pattern present in mCHERRY-RALF22_{mature} kymographs or from a
796 linear ROI drawn along the shank or tip RH CW. The obtained intensity data was detrended in
797 AutoSignal 1.7 (Systat Software) by subtracting a cubic fit. Given that we were interested in the
798 spatial frequency pattern represented by the spacing between individual microdomains, we used
799 Fourier filtering to remove lower frequency oscillation (<0.6Hz) patterns associated with
800 differences in fluorescence intensity along longer stretches of CW. We then generated a
801 continuous wavelet time-frequency spectrum for each trace. The spatial frequency (mCHERRY-
802 RALF22_{mature} fluorescence cycles. μm^{-1}) -power distributions of all RHs were averaged and a
803 gaussian distribution was fitted to get quantitative data on the mCHERRY-RALF22_{mature}
804 microdomain/ring spacing. The ring spacing was calculated as such: ring spacing (μm)= $1 / \text{spatial}$
805 $\text{frequency (mCHERRY-fluorescence cycles.}\mu m^{-1})$. Similarly, the predominant period characterizing
806 the oscillatory growth rate pattern was extracted from the power distributions that described
807 the continuous wavelet time-frequency spectrum of the growth rate oscillograms.
808 Based on the mCHERRY-RALF22_{mature}, 2F4, LM20 and PAM1 Z-stacks, the lateral CW was
809 reconstructed using the 3D project plugin. Colocalization was assessed using the Costes
810 threshold regression method represented in the Coloc2 plugin with 50-100 randomisations⁷⁶.

811 Calcium assay

812 To quantify the systemic $[Ca^{2+}]_{cyt}$ response in trichoblast cell files upon RALF22 treatment, we
813 generated Col-0 plants expressing the BRET-based GFP-aequorin Ca^{2+} -indicator G5A⁵⁴ under the

814 control of the RH-specific EXPANSINa7 promoter. Col-0 x pEXPa7::G5A plants were grown in
815 liquid medium in 96-well microplates and incubated overnight in the presence of coelenterazine,
816 as previously described⁵⁴. Following injection of 200nM RALF22_{mature}, RALF22^{R82A,R90A,R100A} or
817 RALF22^{Y75A,Y78A} the luminescence was quantified for 20min using a TriStar LB 941 Multimode
818 microplate reader equipped with automated well-by-well injection (Berthold Technologies). To
819 investigate the importance of HG methylesterification, plants were preincubated with 32μM
820 EGCG for 30min, 1h, 2h, 3h or overnight prior to RALF22 treatment. The [Ca²⁺]_{cyt} response was
821 compared between treatments by extracting the area under the curve.

822 Pectin and recombinant protein production

823 Synthetic RALF22_{mature} (AQKKYISYGAMRRNSVPCSRRGASYINCQ~~R~~GAQANPYSRGCSTITRCRR),
824 RALF22^{R82A,R90A,R100A} (AQKKYISYGAM~~R~~NSVPC~~S~~ARGASYINCQ~~A~~GAQANPYSRGCSTITRCRR) and
825 RALF22^{Y75A,Y78A} (AQKKA~~I~~SAGAMRRNSVPCSRRGASYINCQ~~R~~GAQANPYSRGCSTITRCRR) peptides were
826 chemically synthesized (ProteoGenix, Schiltigheim, France).

827 LRX1-RALF22, FER_{eecd}, ERU_{eecd} and LLG1 were produced in insect cells as previously described²⁹.
828 Codon-optimized synthetic genes for expression in *Spodoptera frugiperda* (Invitrogen, GeneArt),
829 coding for *Arabidopsis thaliana* LLG1 (residues 24-144, At5g56170), ERU_{eecd} (residues 28-425,
830 At5g61350), FER_{eecd} (residues 1-447, At3g51550), LRX2 (residues 1 to 385; At1g62440), LRX1
831 (residues 28-404, At1g12040) and RALF22_{mature} (residues 71-119, At3g05490) domains were
832 cloned into a modified pFastBac vector (Geneva Biotech), with a native, 30K⁷⁷ or BIP (native
833 signal peptide of *Drosophila melanogaster*) signal peptide; and a TEV (tobacco etch virus
834 protease) cleavable N- or C-terminal StrepII-9xHis tag. *Trichoplusia ni* Tnao38 cells^{78,79} were
835 infected with LLG1, ERULUS, FERONIA or co-infected with LRX1-RALF22 or LRX2-RALF22 with a
836 multiplicity of infection (MOI) of 3 and incubated 26 hours at 28°C and 48 hours at 22°C at 110
837 rpm. The secreted proteins were purified from the supernatant by sequential Ni²⁺ or StrepII
838 affinity chromatography. Ni²⁺ (HisTrap excel, Cytiva) and StrepII (Strep-Tactin Superflow high
839 capacity, IBA Lifesciences) columns were equilibrated in 25mM KPi pH 7.8, 500mM NaCl and
840 25mM Tris pH 8.0, 250mM NaCl, 1mM EDTA respectively. The tags were cleaved with His-tagged
841 TEV protease at 4°C overnight and removed by a second Ni²⁺ affinity chromatography step.
842 Proteins were further purified by SEC on a Superdex 200 Increase 10/300 GL column (Cytiva)
843 equilibrated in 20mM citrate pH 5.0, 150mM NaCl. Proteins were concentrated using Amicon
844 Ultra concentrators (Millipore, molecular weight cut-off 3,000, 10,000 and 30,000), and SDS-
845 PAGE was used to assess the purity and integrity of the different proteins.

846 High and low methoxyl pectin samples were obtained from CPKelco (Denmark). The degree of
847 pectin methylesterification was determined by Fourier transform infrared spectroscopy (FT-IR)
848 using an IS50 spectrometer (ThermoFisher Scientific, Courtaboeuf, France). Pectin solutions were
849 prepared in demineralised water at a concentration of 1 g.L⁻¹, after which the pH was adjusted to
850 pH 6.0, for complete ionization of the carboxylic acid groups. 100μL of solution were put on a
851 CaF₂ support and dried overnight at 35°C. The IR transmittance was measured at wavenumbers
852 1800 cm⁻¹ to 800 cm⁻¹ at a resolution of 2 cm⁻¹. 200 scans were run per sample and averaged to
853 obtain mean spectra. The spectra were baseline corrected, and preprocessed (OPUS and
854 TheUnscrambler softwares). The ratio (X) of the absorption intensity of the bands around
855 1740cm⁻¹ (carbonyl (C = O) stretching) and 1600–1630cm⁻¹ (carboxylate (COO⁻) stretching) was
856 fitted into a calibration equation (Y = 136.86X + 3.987 to calculate the degree of
857 methylesterification (Y)⁸⁰. The analysis was performed in duplicate.

858 The monosaccharide composition was determined by liquid-gas chromatography. Approx. 5mg
859 of pectin powder was hydrolyzed in 2M H₂SO₄ for 2 hours at 100°C in the presence of inositol as
860 internal standard. Sugars were then reduced, acetylated and analyzed as alditol acetates⁸¹ by
861 liquid-gas chromatography (Perkin Elmer, Clarus 580, Shelton, USA) mounted with a DB-225
862 fused-silica capillary column (J&W Scientific, Folsom, CA, USA, at 207°C with H₂ as carrier gas). A

863 standard solution containing the individual neutral monosaccharides (arabinose, rhamnose,
864 glucose, xylose, galactose and mannose) was treated similarly for calculation of the
865 monosaccharide recovery rates. The analysis was performed in triplicate. The results were
866 expressed as anhydrous sugar to take into account their polysaccharide form in the starting
867 sample. Uronic acids were quantified with the automated colorimetric *m*-hydroxybiphenyl
868 method⁸². The analysis was performed in triplicate.

869 Molecular interaction assays

870 We used microscale thermophoresis to identify potential receptor-RALF22 and RALF22-pectin
871 interactions. FER_{ecd}, ERU_{ecd} or LLG1, purified from insect cells, were labeled with the amine-
872 reactive dye NT-647 Red-NHS (NanoTemper, Munich, Germany) according to the manufacturer's
873 instructions. Prior to interaction analysis, protein quality was assessed using a Tycho NT.6
874 (NanoTemper, Munich, Germany). Labeled protein was dissolved in MST buffer (50mM Tris-HCl
875 pH 7.8, 150mM NaCl, 10mM MgCl₂, 0.05% Tween-20). Wild-type or mutant RALF22 was titrated
876 (starting at 100μM) against 20nM of LLG1, FER_{ecd} or ERU_{ecd} and loaded into premium coated
877 Monolith NT.115 MST capillaries (NanoTemper, Munich, Germany). For the ternary LLG1-
878 RALF22-FER_{ecd} complex, LLG1 (40nM) and wild-type or mutant RALF22 (5μM) were preincubated
879 on ice for 15min and subsequently titrated against 5μM of FER_{ecd}. For RALF22-pectin
880 interactions, wild-type or mutant RALF22 (200μM) was titrated against 40nM Alexa Fluor 647-
881 labeled OG7-13⁴⁷. Following 10min incubation at RT, the capillaries were subjected to
882 thermophoresis using a Monolith NT.115 (NanoTemper) at medium MST power and a LED
883 excitation power of 80%. Data were withheld at a S/N >5 and the lack of protein aggregation or
884 ligand-induced initial fluorescence changes. MST data were analyzed using MO. Affinity analysis
885 v2.2.4 NT (NanoTemper).

886 Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

887 QCM-D was carried out with a "Q-Sense Analyser" (Biolin Scientific, Gothenburg, Sweden) using
888 gold-coated SiO₂ base sensors (QX301), spin-coated with 1% polyallylamine hydrochloride
889 (PAH, Sigma-Aldrich, product number 283223) dissolved in water. Using a peristaltic pump a
890 0.1% pectin solution, in acetate buffer (10mM Na-Acetate pH 5.6, 10mM NaCl), was flown over
891 the PAH layer over the period indicated by the first boxed area in Figs. 3D-F and 5H,I. After a
892 washing step with buffer, protein at a concentration of 1μg/ml (wild-type or mutant RALF22) or
893 10μg/ml (LRX2-RALF22), was flown over the pectin layer for the period indicated by the second
894 boxed area in Figs. 3D-F and 5H,I, followed by a washing step. Data presented are frequency (ΔF)
895 and dissipation rate (ΔD) of the 3rd harmonic resonant frequency of the base gold-coated
896 sensors. Before reutilization, sensors were cleaned with a "Piranha" solution (7 vol 95% H₂SO₄
897 added to 3 vol 30% H₂O₂; respect the order!) using the following protocol: the quartz was
898 incubated (4 in a holder) for 3-5min in Piranha solution. Next, the holder with the quartz was
899 moved to two successive baths with MilliQ water. Then, each quartz was individually rinsed with
900 MilliQ water and dried under a nitrogen stream. If traces still appeared on the surface during
901 drying, rinsing and drying steps were repeated.

902

903 Protein structure modelling

904 The crystal structure of the RALF4 mature peptide (residues 59 to 110; PDB: 6TME)²⁹ was used to
905 build a homology model of the mature RALF22 peptide (residues 71 to 119) through the Swiss-
906 Model homology-modeling server (<https://swissmodel.expasy.org/>). The docking of LRX2
907 (PDB:6QXP) and the AlphaFold2 model of LRX1 with the homology model of RALF22 was
908 performed using the Hawdock server⁸³. The N-terminal region of RALF22 (74-88) was docked

909 into the LLG2 structures (PDB: 6A5E), using the Flexpepdock web-server^{84,85}. The FER receptor
910 was then superimposed according to the crystal structure PDB: 6A5E.

911 Statistics

912 Statistics were performed in R⁸⁶. All data are represented as mean±SEM, and n indicates the number
913 of independent replicates. Significance ($\alpha=0.05$) was assessed by two-way analysis of variance
914 (ANOVA, parametric) using linear mixed-effects models followed by a TukeyHSD (for pairwise
915 statistical comparison), or by a Kruskal-Wallis test (non-parametric). Normality or deviations thereof
916 were assessed with the Shapiro Wilkison test (non-normal distribution < W=0.95< normal
917 distribution) and by plotting the corresponding linear model's residual values.

918

919 movie legends

920

921 movie 1. RALF22 loss-of-function root hairs display aberrant growth and frequent bursting.
922 Representative 2h timelapse acquisitions of growing Col-0 and *ralf22-2* (-/-) root hairs.

923 movie 2. Exogenous RALF22 supplementation induces a FERONIA-dependent growth arrest/signaling
924 response and a FERONIA-independent increase in cell wall porosity. Representative longitudinal
925 optical section timelapse acquisitions (10min) of growing Col-0 and *fer-4* RHs expressing the $[Ca^{2+}]_{cyt}$
926 sensor GCaMP3, in the presence of the dextran coupled dyes FITC (110kDa dextran, pH-sensitive) and
927 TRITC (20kDa dextran, pH-insensitive). RHs were imaged in a microfluidics chip for 4min prior to
928 addition of 5 μ M RALF22. The RHs response was followed for another 6 min (scale bar=5 μ m).

929 movie3. RALF22 is secreted to the root hair cell wall where it forms immobile periodic microdomains.
930 (A) Representative longitudinal optical section timelapse acquisition (10min) of a growing *ralf22-2* x
931 *pRALF22::mCHERRY-RALF22_{mature}* RH showing mCHERRY-RALF22_{mature} localization to the entire RH CW.
932 Corresponding kymograph showing the immobility of secreted mCHERRY-RALF22_{mature}
933 microdomains (shown as vertical red lines) throughout the acquisition (scale bar=5 μ m). (B) RALF22
934 microdomains form in the growing RH dome. Consecutive timelapse frames of the growing tip have
935 been aligned to allow visual tracking of mCHERRY-RALF22_{mature} microdomains as they move from tip
936 to shank as the tip grows forward. The concave shape of the growing dome was straightened to
937 generate a kymograph. Red lines in the kymograph depict mCHERRY-RALF22_{mature} microdomains,
938 which originate at the very apex and remain immobile as they move towards the shank (scale
939 bar=5 μ m).

940 movie 4. RALF22 is secreted at the growing root hair tip. Representative longitudinal optical section
941 timelapse acquisition (10 min) of a growing *ralf22-2* x *pRALF22::mCHERRY-RALF22_{mature}* RH. After
942 4min of steady state growth, mCHERRY-RALF22_{mature} fluorescence was bleached in a ROI in the tip
943 and shank. Fluorescence Recovery After Photobleaching was followed for an additional 6min. Rapid
944 mCHERRY-RALF22_{mature} fluorescence recovery was observed in the tip, but not in subapical regions
945 (scale bar=5 μ m).

946 movie 5. The *lrx1-1/2-1* root hair phenotype is indistinguishable from *ralf22-2*. Representative 2h
947 timelapse acquisitions of growing Col-0, *ralf22-2* and *lrx1-1/2-1* RHs.

948 movie 6. Pectin demethylesterification is crucial for root hair growth. Representative longitudinal
949 optical section timelapse acquisitions (10 min) of growing Col-0 RHs expressing the $[Ca^{2+}]_{cyt}$ sensor
950 GCaMP3 in the of dextran coupled FITC (110kDa dextran, pH-sensitive) and propidium iodide (CW
951 charge reporter). Steady state RH growth was imaged for 4min prior to treatment with the Pectin
952 Methylesterase (PME) inhibitor EGCG (100 μ M) or growth medium (mock). The RH's response was
953 followed for an additional 6min.

954 movie 7. LRX1/2 is required for RALF22 secretion. Representative longitudinal optical section
955 timelapse acquisitions (10min) of growing *ralf22-2* x *pRALF22::mCHERRY-RALF22_{mature}* and *lrx1-1/2-1* x
956 *pRALF22::mCHERRY-RALF22_{mature}* RHs illustrating the accumulation of mCHERRY-RALF22_{mature} in
957 intracellular compartments in *lrx1-1/2-1* RHs.

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