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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)<sup>1</sup>, 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)<sup>2</sup>. These properties  
49 include the remarkable ability to expand while simultaneously resisting the tensile forces imposed by  
50 the turgor pressure on the CW<sup>2</sup>.

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 cells<sup>3</sup>. HGs form the most abundant class of pectins, reaching up to 50% of the total CW  
54 polymer content<sup>4</sup>. 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 patterns<sup>2</sup>. *In vivo* studies have associated HG demethylesterification with  
57 either the cessation or promotion of cell expansion, depending on the context<sup>5</sup>. Calcium (Ca<sup>2+</sup>)-  
58 mediated crosslinking of polyanionic HG has been proposed as a driver for CW stiffening and growth  
59 restriction<sup>6,7</sup>. Growth promotion instead, has been proposed to occur upon the exchange of load-  
60 bearing Ca<sup>2+</sup> crosslinks with newly generated pectate, pectin swelling, or more complex scenarios  
61 involving HG turnover or feedback signaling<sup>8-15</sup>. 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 structure<sup>16</sup>. 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 matrices<sup>16</sup>. 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 neurons<sup>16</sup>.

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 crosslinks<sup>17-20</sup>. 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*<sup>21,22</sup>. 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 signaling<sup>23</sup>. 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 Ca<sup>2+</sup> channel of the MILDEW RESISTANCE LOCUS O (MLO) family<sup>24-27</sup>. Related pathways are  
87 required for sperm release from pollen tubes, CW integrity control, stress responses or  
88 mechanosensing<sup>28</sup>. 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<sup>27,29-31</sup>. 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<sup>29,32</sup>.

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  $\text{Ca}^{2+}$  levels, CW pectin charge, Reactive Oxygen  
97 Species (ROS) levels and cytosolic and apoplastic  $\text{pH}^{33}$ . 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)<sup>36</sup>. 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<sup>24,37,38</sup>. Like other RALFs, *RALF22* contains a  
127 propeptide sequence that is cleaved off by subtilisin-like serine proteases during protein  
128 maturation<sup>39,40</sup>. The mature *RALF22* protein harbors two disulfide cysteine-bridges and a  
129 conserved YISY motif that is critical for receptor binding (Fig. 2A)<sup>23,39</sup>. 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 morphogenesis<sup>41-43</sup>. We recombinantly expressed LLG1 and the ERU and FER  
132 ectodomains (ERU<sub>ecd</sub>, FER<sub>ecd</sub>) 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<sub>ecd</sub> or FER<sub>ecd</sub> alone (Fig. S2C). Preincubated LLG1-RALF22,  
137 however, formed a high affinity ( $118.02 \pm 59.01 \text{nM}$ ) ternary complex with FER<sub>ecd</sub>, but not with  
138 ERU<sub>ecd</sub> (Fig. 2C-D, Fig. S2C). Next, we substituted both tyrosines with alanines in the conserved  
139 *RALF22* YISY motif (*RALF22*<sup>Y75A, Y78A</sup>) that mediates receptor binding<sup>23</sup>. Compared to wild type  
140 *RALF22*, we observed a 4.7-fold decrease in the affinity of *RALF22*<sup>Y75A, Y78A</sup> for LLG1

141 (28.48±2.43μM) (Fig. 2B). In addition, LLG1-RALF22<sup>Y75A,Y78A</sup> no longer formed a ternary complex  
142 with FER<sub>ecd</sub> (Fig. 2C). Together, these results show that RALF22, like RALF23<sup>23</sup>, binds to LLG1 and  
143 nucleates the formation of a ternary LLG1-RALF22-FER<sub>ecd</sub> 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<sub>ext</sub>) and  
147 intracellular calcium ([Ca<sup>2+</sup>]<sub>cyt</sub>). To this end, we grew Col-0 and FERONIA loss-of-function (*fer-4*)  
148 seedlings expressing the cytosolic Ca<sup>2+</sup> indicator GCaMP3<sup>44</sup> 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<sup>45,46</sup>. 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<sub>ext</sub> (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, [Ca<sup>2+</sup>]<sub>cyt</sub> and pH<sub>ext</sub> (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 [Ca<sup>2+</sup>]<sub>cyt</sub> (Fig. 2E, H) and an increase in pH<sub>ext</sub> (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 [Ca<sup>2+</sup>]<sub>cyt</sub> and pH<sub>ext</sub> (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, [Ca<sup>2+</sup>]<sub>cyt</sub> and pH<sub>ext</sub> 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)<sup>47</sup>. We observed a robust interaction with a Kd of 3.03±0.53μM (Fig. 3B). The charge  
177 dependency of the interaction is suggested by the 3.7-fold reduction in affinity (Kd =  
178 11.1±2.65μM) for a mutant peptide RALF22<sup>R82A,R90A,R100A</sup> 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)<sup>48</sup>. 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  $\Delta F_{\text{pectin}}$  and increase in  $\Delta D_{\text{pectin}}$ , 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  $\Delta F_{\text{RALF22}}$  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  $\Delta D_{\text{RALF22}}$  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<sup>49,50</sup>. This  
200 interaction was stable under the experimental conditions used since no important changes in  $\Delta F$   
201 and  $\Delta D$  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  $\Delta F_{\text{RALF22}}$  of  
204  $38.98 \pm 4.27\%$ , decrease in  $\Delta D_{\text{RALF22}}$  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<sup>R82A,R90A,R100A</sup> (increase in  $\Delta F$  of  
206  $3.53 \pm 0.24\%$ , decrease in  $\Delta D$  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<sup>16,49,50</sup>. 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 proteins<sup>16</sup>. 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<sub>mature</sub>*; see Star  
220 Methods). *Ralf22-2 x pRALF22::mCherry-RALF22<sub>mature</sub>* 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  $11.5 \pm 0.9$ -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  $1.34 \pm 0.08$

238  $\mu\text{m}$  (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<sup>23,27,29-31</sup>. In RHs, LRX1 and LRX2 have partially redundant roles in  
257 sustaining RH growth<sup>51</sup>. 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<sub>LRR</sub>, PDB: 6QXP)<sup>29</sup> and  
261 an AlphaFold2 model of the corresponding LRX1<sub>LRR</sub> domain (Fig. 5C). RALF22<sub>mature</sub> was modelled  
262 using the crystal structure of RALF4 (PDB: 6TME) as a reference. Similar to the LRX8<sub>LRR</sub>-RALF4  
263 complex<sup>29</sup>, we found that the conserved binding pockets of LRX2<sub>LRR</sub> and LRX1<sub>LRR</sub> could  
264 accommodate RALF22<sub>mature</sub> (Fig. 5C, LRX2<sub>LRR</sub>-RALF22; total energy score =  $-66.43 \text{ kcal} \cdot \text{mol}^{-1}$ ; root  
265 mean square deviation =  $0.760 \text{ \AA}$ ). LRX1<sub>LRR</sub>-RALF22 and LRX2<sub>LRR</sub>-RALF22 dimeric complexes could  
266 indeed be purified from insect cells that co-expressed LRX1<sub>LRR</sub> or LRX2<sub>LRR</sub> with RALF22 (Fig. 5D).  
267 These complexes were extremely stable as shown by the inability of LRX1<sub>LRR</sub>-RALF22 to dissociate  
268 at pH 2.0 (Fig. 5D, E) and their  $92.3^\circ\text{C}$  (LRX1<sub>LRR</sub>-RALF22) and  $83.8^\circ\text{C}$  (LRX2<sub>LRR</sub>-RALF22) dissociation  
269 temperatures in thermal shift assays (Fig. 5F). These results suggest that the LRX1<sub>LRR</sub>-RALF22 and  
270 LRX2<sub>LRR</sub>-RALF22 are bona fide complexes with even higher stability than the previously described  
271 LRX8<sub>LRR</sub>-RALF4 pollen expressed complex<sup>29</sup>.

272 To study the LRX1-RALF22 interaction *in planta*, we introduced *pRALF22::mCHERRY-RALF22<sub>mature</sub>*  
273 into *lrx1-1* plants that express a functional cMyc-tagged LRX1 (*lrx1-1 x pLRX1::cMyc-LRX1*<sup>52</sup>).  
274 cMyc-LRX1 also formed rings perpendicular to the RH's longitudinal axis, that colocalized to a  
275 great extent with mCHERRY-RALF22<sub>mature</sub> (Fig. 5G, H; Costes p-value > 0.95; thresholded Manders'  
276 coefficient (tM)<sub>mCHERRY-RALF22@cMyc-LRX1</sub> =  $0.71 \pm 0.22$ ; tM<sub>cMyc-LRX1@mCHERRY-RALF22</sub> =  $0.70 \pm 0.21$ ; 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<sub>LRR</sub>-RALF22 complex. A  
282 possible pectin interaction is suggested by the 3D crystal structure of the LRX8<sub>LRR</sub>-RALF4 dimer, in  
283 which RALF4 displays a highly positively-charged and surface-exposed patch of amino acids<sup>29</sup>.  
284 Homology modeling shows that this is also true for the LRX1<sub>LRR</sub>-RALF22 complex, albeit with a  
285 less pronounced surface charge compared to LRX8<sub>LRR</sub>-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<sub>LRR</sub>-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  $\text{RALF22}$  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  $\text{RALF22}$  decrease for DM75 of  $22.54 \pm 7.37\%$ ,  $n=6$ ), indicating that LRX1<sub>LRR</sub>-RALF22, like free RALF22,  
293 promoted the stiffening of the pectin layers (Fig. 5K). Concerning the LRX1<sub>LRR</sub>-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<sub>LRR</sub>-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  $\Delta D$  is presumably the resultant of the stiffening effect of  
298 the LRX1<sub>LRR</sub>-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<sub>LRR</sub>-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  $[\text{Ca}^{2+}]_{\text{cyt}}$ ,  $\text{pH}_{\text{ext}}$  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 CW<sup>53</sup>,  
310 and high amplitude  $[\text{Ca}^{2+}]_{\text{cyt}}$  and  $\text{pH}_{\text{ext}}$  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 PMEs<sup>34</sup>. 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 pEXPa7<sup>54</sup>. 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 EC<sub>50</sub> for root growth inhibition ( $32 \mu\text{M}$ <sup>55</sup>), 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<sub>mature</sub>* RHs with monoclonal antibodies (mABs) directed against different HG epitopes (Fig.  
331 6, Fig. S7). LM20, which labels methylesterified HG<sup>56</sup>, 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<sub>mature</sub>, 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  $\text{Ca}^{2+}$ -crosslinked  
336 demethylesterified HG<sup>57</sup>, 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<sub>mature</sub> (Fig. S7D, E;  
338 Costes p-value>0.95;  $tM_{\text{mCherry-RALF22} \rightarrow 2F4} = 0.56 \pm 0.04$ ;  $tM_{2F4 \rightarrow \text{mCherry-RALF22}} = 0.49 \pm 0.02$ ; n=8). Also  
339 along the shank,  $\text{Ca}^{2+}$ -crosslinked demethylesterified HG was organized in rings, where regions  
340 with a relatively lower mCherry-RALF22<sub>mature</sub> 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 HG<sup>58</sup>, 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<sub>mature</sub> (Fig. 6A-D; Costes p-  
345 value>0.95;  $tM_{\text{mCherry-RALF22} \rightarrow \text{PAM1}} = 0.74 \pm 0.05$ ;  $tM_{\text{PAM1} \rightarrow \text{mCherry-RALF22}} = 0.74 \pm 0.04$ ; 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<sub>mature</sub> 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  $\text{Ca}^{2+}$ -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<sub>mature</sub> (*lrx1-1/2-1*  
360 *x pRALF22::mCherry-RALF22<sub>mature</sub>*, 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<sub>mature</sub> 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<sub>mature</sub>* line (Fig.  
365 6G, H, movie 7). Interestingly, in the absence of LRX1 and LRX2, mCherry-RALF22<sub>mature</sub> 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<sub>mature</sub> 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;  $tM_{\text{mCherry-RALF22} \rightarrow \text{PAM1}} = 0.25 \pm 0.08$ ;  
372  $tM_{\text{PAM1} \rightarrow \text{mCherry-RALF22}} = 0.19 \pm 0.07$ ; 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 partners<sup>29</sup> 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<sub>LRR</sub>-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<sub>LRR</sub>-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<sup>1,59</sup>, or acetyl<sup>60</sup> or xylosyl<sup>61</sup> substitution motifs. In addition, the  
404 LRX1<sub>LRR</sub>-RALF22 dimer may promote polymer crosslinking, thus reducing the water binding  
405 capacity (Fig. 3D-G, Fig. 5I-K, Fig. S4A, B)<sup>62</sup>. 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 neurons<sup>16</sup>. 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 properties<sup>63</sup>. 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 previously<sup>64-66</sup>. For instance, stretched epidermal cells

429 upon root bending displayed biphasic cytosolic  $\text{Ca}^{2+}$  responses, with a rapid transient and a  
430 slower more sustained component. Only the latter disappeared in *llg1* and *fer* mutants<sup>65</sup>,  
431 whereas the initial transient might come from another mechanosensor, such as a  
432 mechanosensitive  $\text{Ca}^{2+}$ -permeable channel<sup>67</sup>. 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 ROPGEF14<sup>65</sup>. 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 PMEs<sup>34</sup>. 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 cellulose<sup>68</sup>, xyloglucan<sup>69</sup> and expansins<sup>70</sup> 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 resolution<sup>11</sup>, should provide more insights into this fascinating CW  
463 assembly process in RHs and most likely in other cell types in plants.

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## 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<sup>Y75A,Y78A</sup>  
514 (n=3). (C) Binding affinity between the FERONIA ectodomain (FER<sub>ecd</sub>) and fluorescently labelled LLG1  
515 preincubated with RALF22 (n=3) or RALF22<sup>Y75A,Y78A</sup> (n=3). MST values are represented as the average  
516 normalized change in fluorescence (%) ± SEM. (D) Homology model of the LLG2-RALF22-FER<sub>ecd</sub>  
517 complex. The N-terminal region of RALF22 was docked into the LLG2 structure (PDB:6A5E). FER<sub>ecd</sub> 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, [Ca<sup>2+</sup>]<sub>cyt</sub> (cytosolic GCaMP3 fluorescence  
521 intensity), pH<sub>ext</sub> (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<sup>R82A, R90A, R100A</sup>)  
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  $\Delta F$  and  $\Delta D$  QCM-D traces for a pectin layer with a  
540 degree of methylesterification of 75% (D) or 31% (E) treated with  $1\mu g.mL^{-1}$  RALF22 or DM31 pectin  
541 treated with  $1\mu g.mL^{-1}$  RALF22<sup>R82A, R90A, R100A</sup>. Grey zones highlight the intervals during which pectin or  
542 RALF were applied (cfr. Fig. S4A,B). (G) Quantification of the % change in  $\Delta F$  and  $\Delta D$  upon RALF  
543 treatment for the conditions presented in D-F (DM75 + RALF22, n=14; DM31 + RALF22, n=14; DM31 +  
544 RALF22<sup>R82A, R90A, R100A</sup>, 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<sub>mature</sub>* roots (scale bar=500 $\mu m$ ) and RHs (scale bar=100 $\mu m$ ). (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<sub>mature</sub>* RH (scale bar=8 $\mu m$ ). ROIs showing the presence of mCHERRY-RALF22<sub>mature</sub> microdomains  
553 in the RH CW (scale bar=2 $\mu m$ ). (D) maximal projection kymograph corresponding to a 10min  
554 timelapse acquisition of a growing *ralf22-2 x pRALF22::mCHERRY-RALF22<sub>mature</sub>* RH. Vertical red lines  
555 represent mCHERRY-RALF22<sub>mature</sub> microdomains that remained stationary in the CW throughout the  
556 acquisition. The longitudinal fluorescence pattern for quantification of the mCHERRY-RALF22<sub>mature</sub>  
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<sub>mature</sub> 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<sub>mature</sub>* RH before, upon and after photobleaching of mCHERRY-RALF22<sub>mature</sub> in ROIs in the CW of  
562 the expanding dome, below the tip and in the shank (scale bar=5 $\mu m$ ). Graphs showing mCHERRY  
563 fluorescence recovery at the apex only. (G) representative timeseries showing that RH growth rate  
564 and apical mCHERRY-RALF22<sub>mature</sub> fluorescence oscillate in antiphase. (H) frontal and lateral high-  
565 resolution z-projections of mCHERRY-RALF22<sub>mature</sub> organization in the RH CW, illustrating the  
566 formation of regularly spaced mCHERRY-RALF22<sub>mature</sub> rings (scale bar=2 $\mu m$ ). (I) Representative high  
567 resolution longitudinal optical section of a representative growing RH (scale bar=5 $\mu m$ ) showing  
568 mCHERRY-RALF22<sub>mature</sub> microdomains in the tip and the shank (scale bar=4 $\mu m$ ). Comparative analysis  
569 of mCHERRY-RALF22<sub>mature</sub> 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 $\mu$ m) and close-ups (scale bar=100 $\mu$ 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 $\text{\AA}$  comparing 342 pairs of  
578 corresponding C $\alpha$  atoms between the two proteins. Full molecular docking model of RALF22 (green)  
579 with LRX2<sub>LRR</sub> (PDB: 6QXP; grey) (top right). Enlarged view of the peptide docking models showing  
580 RALF22 (green) in the LRX2<sub>LRR</sub> and LRX1<sub>LRR</sub> (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<sub>LRR</sub>-RALF22 (pH 5.0 and 2.0) and LRX2<sub>LRR</sub>-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<sub>LRR</sub>-RALF22 (pH 5.0 and 2.0) and LRX2<sub>LRR</sub>-  
585 RALF22 (pH 5.0). (F) thermoshift assay of LRX8<sub>LRR</sub>-RALF4 (control), LRX1<sub>LRR</sub>-RALF22 and LRX2<sub>LRR</sub>-  
586 RALF22. LRX1<sub>LRR</sub>-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<sub>mature</sub>* CW in which LRX1 was labeled with a green  
589 fluorescent anti-cMyc antibody (scale bar=5 $\mu$ m). Dots represent cMyc-LRX1 (green) and mCHERRY-  
590 RALF22<sub>mature</sub> microdomains (red). Composite images and magnifications (scale bar=1 $\mu$ m) show  
591 colocalization (yellow) between LRX1 and RALF22. (H) Representative lateral z-projections of cMyc-  
592 LRX1 (green) and mCHERRY-RALF22<sub>mature</sub> (red) organization and colocalization (yellow) in the RH CW  
593 (scale bar=5 $\mu$ m). cMyc-LRX1 colocalizes with RALF22<sub>mature</sub> rings in the CW. (I,J) representative  $\Delta$ F and  
594  $\Delta$ D traces of DM75 (I) and DM31 (J) treated with 10  $\mu$ g.mL<sup>-1</sup> of LRX1<sub>LRR</sub>-RALF22. (K) Quantification of  
595 the % change in  $\Delta$ F and  $\Delta$ D (relative to the pectin-induced change) upon LRX1<sub>LRR</sub>-RALF22 treatment  
596 for the conditions presented in I and J (DM31 + LRX1<sub>LRR</sub>-RALF22, n=11; DM75 + LRX1<sub>LRR</sub>-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<sub>mature</sub> in the RH tip and  
602 shank. (A) longitudinal optical sections of a PAM1 (green) and mCHERRY-RALF22<sub>mature</sub> labeled RH  
603 (scale bar=10 $\mu$ 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<sub>mature</sub>  
605 colocalize along the growing dome (scale bar=2.5 $\mu$ 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<sub>mature</sub> (red) (scale bar=1 $\mu$ m). (D) lateral z-projections of the  
608 RH shank CW showing the formation of colocalized PAM1 and mCHERRY-RALF22<sub>mature</sub> circumferential  
609 rings (scale bar=5 $\mu$ m). (E) representative longitudinal optical sections of *ralf22-2* RHs labeled with  
610 PAM1 (demethylesterified HG), 2F4 (Ca<sup>2+</sup>-crosslinked demethylesterified HG) and LM20  
611 (methylesterified HG) (scale bar=10 $\mu$ m). Asterisks indicate expelled intracellular debris as a result of  
612 cell bursting. (F) quantification of the labeling intensities for the PAM1 (n<sub>col-0</sub>=12; n<sub>PAM1</sub>=16), 2F4 (n<sub>col-0</sub>=  
613 8; n<sub>2F4</sub>=9) and LM20 epitopes (n<sub>col-0</sub>=19; n<sub>PAM1</sub>=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<sub>mature</sub> (red). Yellow indicates colocalization in the  
618 composite frame (scale bar=5 $\mu$ m). (H) Quantification of the PAM1 (green) and mCHERRY-RALF22<sub>mature</sub>  
619 (red) CW labeling intensities in the tip and shank of *lrx1-1/2-1* x *pRALF22::mCHERRY-RALF22<sub>mature</sub>* and  
620 complemented *ralf22-2* x *pRALF22::mCHERRY-RALF22<sub>mature</sub>* 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<sub>mature</sub>* RH shank CW showing the fragmented pattern of  
624 demethylesterified HG and RALF22 organization (scale bar=5 $\mu$ 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<sub>mature</sub>*, *lrx1-1/2-1 x*  
654 *pRALF22::mCHERRY-RALF22<sub>mature</sub>* and *lrx1-1 x pLRX1::myc-LRX1/pRALF22::mCHERRY-  
655 RALF22<sub>mature</sub>* lines were generated during this study. *Eru x pERU::ERU-GFP* was generated  
656 previously<sup>42</sup>.

### 657 **Growth conditions**

658 Seeds were surface sterilized and sown on RH growth medium (3mM KNO<sub>3</sub>, 2mM  
659 Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 0.5mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1mg.mL<sup>-1</sup> thiamine, 0.5 mg.mL<sup>-1</sup>  
660 pyridoxine-HCl, 0.5mg.mL<sup>-1</sup> nicotinic acid, 0.56mM myo-inositol, 25mM KCl, 17.5mM H<sub>3</sub>BO<sub>3</sub>,  
661 1mM MnSO<sub>4</sub>·H<sub>2</sub>O, 1mM ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.25mM CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.25mM (NH<sub>4</sub>)<sub>6</sub>MoO<sub>24</sub>·4H<sub>2</sub>O, 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<sup>-1</sup> 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 CaCl<sub>2</sub>, 1mM NaCl, 1% sucrose, 0.5g.L<sup>-1</sup> 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 Browser<sup>71</sup>, 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 plots<sup>36</sup>. For *RALF22*, a trichoblast pseudotime expression profile was generated, showing *RALF22*  
676 expression in function of the trichoblast's distance to the meristem<sup>37</sup>.

### 677 Immunolocalization

678 Four day old Col-0, *ralf22-2*, *ralf22-2 x pRALF22::mCHERRY-RALF22<sub>mature</sub>*, *lrx1-1 x pLRX1::cmyc-*  
679 *LRX1/pRALF22::mCHERRY-RALF22<sub>mature</sub>* and *lrx1-1/2-1 x pRALF22::mCHERRY-RALF22<sub>mature</sub>*  
680 seedlings were grown overnight in microfluidics chips<sup>72</sup> 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 NH<sub>4</sub>Cl 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-Alexa<sup>488</sup>, PAM1; Rabbit anti-His-  
688 DyLight<sup>488</sup>, cMyc-LRX1; anti-c-Myc-Alexa<sup>488</sup>). 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  $\mu$ -slides VI 0.4 filled  
696 with liquid medium<sup>72</sup>. Each channel contained a single plant.

697 Col-0 *x pRALF22::GFP* plants were counterstained by injecting 10 $\mu$ 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 $\mu$ m-2 $\mu$ m.

703 The same system with a 63x HC PL APO CS2 water immersion objective (NA 1.20) was used to  
704 image [Ca<sup>2+</sup>]<sub>cyt</sub>, pH<sub>ext</sub>, 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 $\mu$ L  
706 of unbuffered liquid medium. Prior to imaging, 160 $\mu$ L of medium was removed from each  
707 channel. 60 $\mu$ L of medium containing 0.29 mg.mL<sup>-1</sup> FITC-110kDa dextran and 0.25 mg.mL<sup>-1</sup> TRITC-  
708 20kDa dextran (for *RALF22* treatment) or 0.29 mg.mL<sup>-1</sup> FITC-110kDa dextran and 10 $\mu$ 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<sup>-1</sup>. 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 $\mu$ L of liquid medium containing either 5 $\mu$ M *RALF22* or 100 $\mu$ 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<sub>mature</sub>* and *lrx1-1/2-1 x pRALF22::mCHERRY-RALF22<sub>mature</sub>* 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<sup>-1</sup>.

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<sub>mature</sub>* 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<sub>mature</sub> 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<sub>mature</sub>* 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<sub>mature</sub>* 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 [Ca<sup>2+</sup>]<sub>cyt</sub> BRET sensor we synthesized a construct containing the 535bp promoter of the  
751 *EXPANSINa7* gene and the G5A sequence<sup>54</sup>. 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 reaction<sup>73</sup>. *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 method<sup>74</sup>. To generate the *pRALF22::RALF22<sup>R82A,R90A,R100A</sup>* 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 ASE<sup>75</sup>.

### 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<sub>mature</sub> 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<sub>mature</sub> 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<sub>mature</sub> 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<sub>mature</sub> fluorescence  
795 intensity pattern from the line pattern present in mCHERRY-RALF22<sub>mature</sub> 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<sub>mature</sub> fluorescence cycles. $\mu m^{-1}$ ) -power distributions of all RHs were averaged and a  
803 gaussian distribution was fitted to get quantitative data on the mCHERRY-RALF22<sub>mature</sub>  
804 microdomain/ring spacing. The ring spacing was calculated as such: ring spacing ( $\mu m$ )=  $1 / \text{spatial}$   
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<sub>mature</sub>, 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 randomisations<sup>76</sup>.

## 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 G5A<sup>54</sup> 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 described<sup>54</sup>. Following injection of 200nM RALF22<sub>mature</sub>, RALF22<sup>R82A,R90A,R100A</sup> or  
817 RALF22<sup>Y75A,Y78A</sup> 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 [Ca<sup>2+</sup>]<sub>cyt</sub> response was  
821 compared between treatments by extracting the area under the curve.

## 822 Pectin and recombinant protein production

823 Synthetic RALF22<sub>mature</sub> (AQKKYISYGAMRRNSVPCSRRGASYYNCQ~~R~~GAQANPYSRGCSTITRCRR),  
824 RALF22<sup>R82A,R90A,R100A</sup> (AQKKYISYGAM~~R~~NSVPC~~S~~ARGASYYNCQ~~A~~GAQANPYSRGCSTITRCRR) and  
825 RALF22<sup>Y75A,Y78A</sup> (AQKKAIS~~A~~GAMRRNSVPCSRRGASYYNCQ~~R~~GAQANPYSRGCSTITRCRR) peptides were  
826 chemically synthesized (ProteoGenix, Schiltigheim, France).

827 LRX1-RALF22, FER<sub>eecd</sub>, ERU<sub>eecd</sub> and LLG1 were produced in insect cells as previously described<sup>29</sup>.  
828 Codon-optimized synthetic genes for expression in *Spodoptera frugiperda* (Invitrogen, GeneArt),  
829 coding for *Arabidopsis thaliana* LLG1 (residues 24-144, At5g56170), ERU<sub>eecd</sub> (residues 28-425,  
830 At5g61350), FER<sub>eecd</sub> (residues 1-447, At3g51550), LRX2 (residues 1 to 385; At1g62440), LRX1  
831 (residues 28-404, At1g12040) and RALF22<sub>mature</sub> (residues 71-119, At3g05490) domains were  
832 cloned into a modified pFastBac vector (Geneva Biotech), with a native, 30K<sup>77</sup> 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<sup>78,79</sup> 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 Ni<sup>2+</sup> or StrepII  
838 affinity chromatography. Ni<sup>2+</sup> (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 Ni<sup>2+</sup> 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<sup>-1</sup>, 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 CaF<sub>2</sub> support and dried overnight at 35°C. The IR transmittance was measured at wavenumbers  
852 1800 cm<sup>-1</sup> to 800 cm<sup>-1</sup> at a resolution of 2 cm<sup>-1</sup>. 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<sup>-1</sup> (carbonyl (C = O) stretching) and 1600–1630cm<sup>-1</sup> (carboxylate (COO<sup>-</sup>) stretching) was  
856 fitted into a calibration equation (Y = 136.86X + 3.987 to calculate the degree of  
857 methylesterification (Y)<sup>80</sup>. 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 H<sub>2</sub>SO<sub>4</sub> for 2 hours at 100°C in the presence of inositol as  
860 internal standard. Sugars were then reduced, acetylated and analyzed as alditol acetates<sup>81</sup> 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 H<sub>2</sub> 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 method<sup>82</sup>. 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<sub>ecd</sub>, ERU<sub>ecd</sub> 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 MgCl<sub>2</sub>, 0.05% Tween-20). Wild-type or mutant RALF22 was titrated  
876 (starting at 100μM) against 20nM of LLG1, FER<sub>ecd</sub> or ERU<sub>ecd</sub> and loaded into premium coated  
877 Monolith NT.115 MST capillaries (NanoTemper, Munich, Germany). For the ternary LLG1-  
878 RALF22-FER<sub>ecd</sub> complex, LLG1 (40nM) and wild-type or mutant RALF22 (5μM) were preincubated  
879 on ice for 15min and subsequently titrated against 5μM of FER<sub>ecd</sub>. For RALF22-pectin  
880 interactions, wild-type or mutant RALF22 (200μM) was titrated against 40nM Alexa Fluor 647-  
881 labeled OG7-13<sup>47</sup>. 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 SiO<sub>2</sub> 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 3<sup>rd</sup> harmonic resonant frequency of the base gold-coated  
896 sensors. Before reutilization, sensors were cleaned with a "Piranha" solution (7 vol 95% H<sub>2</sub>SO<sub>4</sub>  
897 added to 3 vol 30% H<sub>2</sub>O<sub>2</sub>; 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)<sup>29</sup> 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 server<sup>83</sup>. The N-terminal region of RALF22 (74-88) was docked

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

## 911 Statistics

912 Statistics were performed in R<sup>86</sup>. 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 $\mu$ M RALF22. The RHs response was followed for another 6 min (scale bar=5 $\mu$ 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<sub>mature</sub>* RH showing mCHERRY-RALF22<sub>mature</sub> localization to the entire RH CW.  
932 Corresponding kymograph showing the immobility of secreted mCHERRY-RALF22<sub>mature</sub>  
933 microdomains (shown as vertical red lines) throughout the acquisition (scale bar=5 $\mu$ 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<sub>mature</sub> 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<sub>mature</sub> microdomains,  
938 which originate at the very apex and remain immobile as they move towards the shank (scale  
939 bar=5 $\mu$ 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<sub>mature</sub>* RH. After  
942 4min of steady state growth, mCHERRY-RALF22<sub>mature</sub> 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<sub>mature</sub> fluorescence recovery was observed in the tip, but not in subapical regions  
945 (scale bar=5 $\mu$ 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 $\mu$ 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<sub>mature</sub>* and *lrx1-1/2-1* x  
956 *pRALF22::mCHERRY-RALF22<sub>mature</sub>* RHs illustrating the accumulation of mCHERRY-RALF22<sub>mature</sub> in  
957 intracellular compartments in *lrx1-1/2-1* RHs.

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