CONFLICTING AUXIN-PHOSPHATE SIGNALS IMPACT ON RSL2 EXPRESSION AND ROS-HOMEOSTASIS LINKED TO ROOT HAIR GROWTH IN *ARABIDOPSIS*

How plant cells regulate their size is one of the most fascinating questions in current plant biology. Root hairs are single plant cells that can expand several hundred-fold their original size and they are an excellent model system for learning about cell size regulation. Root hair size determines the surface area/volume ratio of the whole roots exposed to nutrient and water pools, thereby likely impacting nutrient and water uptake rates. The speed at which they grow is determined by cell-intrinsic factors like hormones (e.g., auxin and ethylene) and external environmental signals like nutrients availability in the soil (e.g., phosphate and nitrates) (Feng et al. 2017; Marzol et al. 2017). The root hair polar growth program is initially triggered by the transcription factors (TFs) of basic helix-loop-helix (bHLH) family RHD6 (ROOT HAIR DEFECTIVE 6) /RSL1 (ROOT HAIR DEFECTIVE 6 LIKE 1) in the initiation phase, and then, activated by the expression of RSL4/RSL2 during the elongation phase (Menand et al. 2007; Yi et al. 2010). Hormones and environmental cues converge to regulate the expression of RSL4, which controls the final root hair cell size (Yi et al. 2011; Lee et al.2013; Datta el al. 2015; Song et al. 2016; Rymen et al. 2017). Although, in this process, other TFs might act in a coordinative manner under specific signals such as Pi-starvation and ethylene as this is the case for EIN3 (ETHYLENE INSENSITIVE 3)/EIL1 (ETHYLENE INSENSITIVE 3-LIKE 1) and LRL3 (LOTUS JAPONICA ROOTHAIRLESS-LIKE1) (Yi et al. 2010; Song et al. 2016, Feng et al. 2017). Recently, it was found that auxin, by releasing several ARFs (e.g., ARF5, ARF7, ARF8 and ARF19) from Aux/IAA proteins, directly activates RSL4 expression and controls root hair growth linked to ROS-homeostasis involving three RESPIRATORY BURST OXIDASE HOMOLOG proteins (e.g., RBOHC,H,J) and four type-III secreted peroxidases (e.g., PER1,44,73) (Mangano et al. 2017). In addition, RSL2 was also involved in the auxin-mediated growth response although it was unclear its mode of action at the molecular level (Mangano et al. 2017). Conversely, high levels of inorganic phosphates (Pi) in the soil (or in the media) are able to strongly repress RSL4 expression linked to polar growth by an unknown mechanism while Pi-starvation results in an extensive outgrowth of root hairs. This response is associated with increased activity of two MYB-transcription factors, PHR1 (PHOSPHATE STARVATION RESPONSE) and PHR1-LIKE1 (PHL1), the homeodomain transcription factor AL6/PER2

1

30 (ALFIN-LIKE6/Pi DEFICIENCY ROOT HAIR DEFECTIVE 2), EIN3/ENL1, and RSL4 (Bustos et al., 2010; Yi

31 et al., 2010; Chandrika et al., 2013; Datta el al. 2015; Song et al. 2016; Feng et al. 2017). Here, we

32 examined by which mechanism root hairs integrate conflicting growth-signals like the repressive

33 high Pi-level clue and a concomitant high auxin exposure that should promote growth and

34 questioned if these complex signals might activate known molecular players in polar growth.

35

36 **Auxin overcome Pi-repression of root hair growth in a ROS-dependent manner**

37 Increased levels of Pi in the media (from 1-20 mM) were able to strongly repress root hair growth as

38 previously reported (Datta et al. 2015; Song et al. 2016) as well as to greatly reduce the Reactive

39 Oxygen Species (ROS) levels in root hair cells in the *Arabidopsis thaliana* model. ROS levels were

40 measured at the root hair cell tip with the cell permeable probe $H_2DCF-DA$ (2',7'-

41 dichlorodihydrofluorescein diacetate) that becomes irreversibly fluorescent under ROS-oxidation.

42 Then, an exogenous auxin supply (100 nM IAA, Indole 3-Acetic Acid) was able to restore root hair

43 growth independently of the level of Pi in the media (**Figure 1A**). In addition, ROS levels were also

44 re-established in the auxin-treated root hairs even in the presence of high-levels of Pi suggesting

45 that both signals, high-Pi levels as well as high-auxin, operated in an opposed manner by a ROS-

46 dependent mechanism (**Figure 1A**). Then, it was tested whether the suppression of ROS-derived

47 from RBOHs activities with VAS2870 (VAS, a specific RBOHs inhibitor) might affect auxin growth-

48 effect in the presence of Pi (**Figure 1B**). VAS treatment was able to revert the auxin growth-effect at

49 the root hair level by down-regulating RBOHs-derived ROS production (**Figure 1B**). A similar

50 repressive effect was obtained when *rbohc* mutant with low-ROS, was incubated with auxin in the

51 presence of high Pi-levels (**Figure 1B**). This confirms that drastic changes in ROS-homeostasis

52 controlled mostly by RBOHC inhibit auxin growth promoting effect. It was previously shown that the

53 over-expression of either RSL4 or Auxin Response Factor 5 (ARF5) under the control of the root hair

54 EXPANSIN 7 promoter (E7) led to high-levels of ROS (Mangano et al. 2017). In addition, E7:RSL4 and

55 E7:ARF5 were more insensitive to high Pi levels that Wt Col-0 and they developed almost regular

56 extended root hairs (**Figure 1C**). This result indicates that high expression of RSL4 or constitutive

57 activated auxin signaling, both are able to partially repress the high levels of Pi-growth effect. To

58 corroborate the ROS measurements performed with the $H_2DCF-DA$ probe as well as to determine if

59   the main ROS molecule involved was hydrogen peroxide (H_2O_2), we measured cytoplasmic H_2O_2

60   ($_{cyt}H_2O_2$) levels using a genetically encoded YFP-based H_2O_2 sensor, HyPer (Mangano et al. 2017).

61   Similar results were obtained for high-levels of Pi that first repressed cytoplasmic H_2O_2 while

62   exogenous auxin was able to trigger an up-regulation of the ROS-signal in the presence of high-Pi in

63   the root hairs (**Figure 1D**). All together indicates that auxin is able to overcome Pi-growth repression

64   and it requires ROS-production to trigger proper polar-growth.

65

66   **Expression of RSL2 is required to bypass Pi-growth repression in the presence of auxin**

67   In order to have insights on the molecular mechanism behind these high Pi-auxin conflicting

68   responses, we tested root hair growth linked to ROS-levels in the absence of RSL2 and RSL4 TFs that

69   were shown to regulate the auxin-mediated root hair growth-response (Datta et al. 2015; Mangano

70   et al. 2017). The double mutant *rsl2 rsl4* is not capable of develop visible root hairs in any condition

71   tested, either under high levels of auxin or ethylene or nutrient deprivation suggesting that both

72   TFs are necessary to run the basic transcriptional machinery to produce root hairs (Mangano et. al

73   2017; Feng et al. 2017). High Pi-level strongly represses the expression of both, RSL2 and RSL4 TFs in

74   the Wt Col-0 roots while auxin treatment up-regulates both TFs, and not only RSL4 as previously

75   indicated (Pires et al. 2013). When *rsl4* (that lacks RSL4 transcripts) was tested, the response was

76   similar to Wt Col-0 root hairs but strongly attenuated indicating that auxin can rescue the negative

77   effect of high-Pi levels, possibly mediated by RSL2 action. In agreement with this idea, when RSL2

78   was lacking (in *rsl2* mutant), there was almost no response associated to the auxin treatment

79   (**Figure 1E**). This indicates that RSL2 mediates the auxin rescue-response in the presence of high Pi-

80   levels. In addition, ROS measurements in Wt Col-0 as well as in *rsl4* and *rsl2* mutants positively

81   correlate with growth responses in all conditions tested (**Figure 1F**). As a complementary approach,

82   levels of RSL2 and RSL4 transcripts were measured in their respective mutant backgrounds (*rsl4* and

83   *rsl2*, respectively) to define if any of these two TFs depends on the presence of the other to be

84   expressed under these conflicting growth conditions (**Figure 1F**). In the *rsl4* mutant background, the

85   expression of RSL2 was down regulated 50% to the levels found in Wt Col-0, while, in *rsl2*

86   background, the level of RSL4 expression was three to four-times lower in all conditions measured

87   (e.g., no Pi or auxin added, high Pi-level, high auxin, or both). This indicates that the absence of

88   RSL2 might affect RSL4 expression, and when RSL4 is lacking, RSL2 transcripts also decrease

89   suggesting a positive feed forward loop between RSL4-RSL2 by a direct or indirect mechanism that

90   requires further investigation.

91

92   Several possible scenarios can now be considered to understand how auxin together with high Pi-

93   levels control root hair growth (**Figure S2**). Since high levels of Pi strongly repress root hair growth

94   and ROS-production, Pi might operate at several regulatory points such as down regulating auxin

95   biosynthesis, auxin-conjugation and transport within the roots towards the trichoblast cells

96   (Velasquez et al. 2016), and then, indirectly affecting RSL4/RSL2 expression. Alternatively, high Pi

97   levels could directly repress RSL4/RSL2 expression by an unknown mechanism. Recently, abscisic

98   acid (ABA) throughout the TF OBP4 (OBF BINDING PROTEIN 4) was able to repress RSL2 (and RSL3)

99   expression indicating that multiple hormonal signals on top of auxin may operate in these cells as

100   previously described (Rymen et al. 2017). In addition, it is plausible that RSL2 and RSL4 would be

101   able to form protein dimers as reported in other several TFs that contains HLH domain (Feller et al.

102   2011), more precisely heterodimers (e.g., RSL2-RSL4) and homodimers (e.g., RSL2-RSL2 and RSL4-

103   RSL4) with slightly different transcriptional downstream target genes. Since it has been shown that

104   RSL4 is able to self-activate by a forward positive-loop (Hwang et al. 2017), we hypothesized that

105   RSL2 would be also capable to the same. In addition, RSL2 would be able to up-regulate RSL4, and

106   vice versa as it was suggested in a previous work (Pires et al. 2013). Further studies are now

107   required to establish the interconnections between both TFs under the different conditions of auxin

108   and Pi tested in this work. Second, since RSL4 is directly activated by ARF5 (and possible several

109   other ARFs including ARF7,8,19) in trichoblast cells (Mangano et. al 2017), we also postulate that

110   RSL2 would be up-regulated by some of these ARFs since auxin triggers its expression two and half-

111   times (**Figure 1E**). In agreement, three consensus Aux-RE sites are found in the regulatory region of

112   RSL2 as possible targets for ARFs binding (not shown). Still how the fine-tune regulation of ARFs on

113   the RSL2-RSL4 is coordinated remains to be discovered. In summary, although it has been shown

114   before that auxin triggers root hair cell elongation while high Pi-levels repress its growth, it was

115   unknown how both conflicting signals might act together. This study identifies a new layer of

116   complexity between RSL2/RSL4 TFs acting on ROS-homeostasis under conflicting growth-signals at

117   the root hair level.

118   **Figure Legend**

119   **Figure 1. Auxin overcomes high-Pi root hair growth-repression by enhancing RSL2 expression**

120   **linked to ROS-homeostasis.**

121   A. Auxin circumvents root hair growth repression imposed by increased levels of Pi by enhancing

122   ROS-production. Root hair length (mean $\pm$ SD) was measured in Wt Col-0 roots non-treated, treated

123   with 100nM IAA (indole 3-acetic acid) or with 5mM inorganic Pi, or both treatments at the same

124   time (on the left). Total ROS levels generated by oxidation of H_2DCF-DA were measured at the root

125   hair tip in different stages of root hair development in the four different treatments (on the right).

126   B. Auxin recovery of root hair growth is dependent on ROS-production. Root hairs treated with 100

127   nM IAA in the presence of high levels of Pi and VAS inhibitor fails to recover its normal growth (on

128   the left). ROS-levels were down regulated in the treated root hairs with 100 nM IAA in the presence

129   of high levels of Pi and VAS inhibitor (on the right). ND= root hairs not detected.

130   C. High levels of ARF5 and RSL4 expression in root hairs cells (EXPANSIN 7 promoter, E7) are able to

131   partially overcome the growth repression imposed by high levels of Pi on cell elongation.

132   Comparisons are made between Wt Col-0 and $_{E7}$:ARF5 or $_{E7}$:RSL4 lines under high levels of Pi.

133   D. Auxin trigger changes in $_{cyt}H_2O_2$ levels detected with HyPer. $_{cyt}H_2O_2$ levels Wt Col-0 root hairs

134   expressing HyPer sensor treated with 5 mM Pi and 100 nM IAA. $_{cyt}H_2O_2$ levels are based on the ratio

135   488/405 nm of HyPer biosensor at the root hair tip over 200 s. On the right, selected kymographs

136   resulting of this analysis only for root hairs of >200 µm in length. Scale bar = 5 µm.

137   E. RSL2 but not RSL4 mediates the auxin recovery response in the presence of high Pi-levels. Root

138   hair length (mean $\pm$ SD) was measured in Wt Col-0, *rsl4* and *rsl2* roots non-treated, treated with

139   100nM IAA (indole 3-acetic acid) or with 5mM inorganic Pi, or both treatments at the same time.

140   ROS-levels were partially recovered in Wt but not in *rsl2* when treated with 100 nM IAA in the

141   presence of high levels of Pi. No ROS was detected in *rsl4* under high levels of Pi. Only root hairs

142   <200 µm in length were analyzed.

143   F. Auxin under high Pi-levels triggers the expression of both, RSL4 and RSL2. Levels of RSL4 and RSL2

144   expression in Wt Col-0 roots non-treated treated with 100nM IAA (indole 3-acetic acid) or with

145   5mM inorganic Pi, or both treatments at the same time (on the left). Levels of RSL4 and RSL2

146   expression in *rsl2* and *rsl4* respectively non-treated treated with 100nM IAA (indole 3-acetic acid) or

6

147 with 5mM inorganic Pi, or both treatments at the same time (on the left). Each qRT-PCR reaction

148 was performed in triplicate, and each experiment was repeated three times using independent

149 preparations of RNA. Comparisons are made in the same genetic background between levels of

150 gene expression (of RSL2 or RSL4) in non-treated samples (no IAA/no Pi) versus treated with IAA or

151 Pi. In addition, Pi-treated samples are compared only to Pi+IAA-treated roots. In Figs 1A-F, *P*-value

152 of one-way ANOVA, (**) $P<0.001$, (*) $P<0.01$. NS= not significant differences. Error bars indicate

153 ±SD from 3 biological replicates.

154 Word count: 544

155

156 **Acknowledgements**

157 We apologize for the inadvertent omission of any pertinent original references owing to space

158 limitations. No conflict of interest is declared.

159

160 **Author's contributions**:

161 S.M performed all the experiments, reviewed the text, figures, and references; S.P.D.J. performed

162 some of the HyPer experiments; E.M. and C.B. reviewed text, references, and figures; J.M.E

163 conceived the project, designed the figures, and wrote the article with contributions of all the

164 authors.

165

166 **Competing financial interest**

167 The authors declare no competing financial interests. Correspondence and requests for materials

168 should be addressed to J.M.E. (Email: jestevez@leloir.org.ar).

169

170 **Financial source**

171 This work was supported by a grant from ANPCyT (PICT2014-0504 and PICT2016-0132) and ICGEB

172 (CRP/ARG16-03 Grant) to J.M.E and a grant from ANPCyT (PICT2016-0491) to S.M.

Silvina Mangano[1], Silvina Paola Denita-Juarez[1], Eliana Marzol[1], Cecilia Borassi[1], and José M. Estevez[1],[†]

[1]Fundación Instituto Leloir and Instituto de Investigaciones Bioquímicas de Buenos Aires (IIBBA-CONICET), Av. Patricias Argentinas 435, Buenos Aires CP C1405BWE, Argentina. TEL: (+54)-115238-7500 EXT. 3206. Email: jestevez@leloir.org.ar. Twitter @EstevezJoseM

[†]Author for correspondence: José M. Estevez

Word count: 1,659

1 Figure

17 references

REFERENCES

Bustos R, Castrillo G, Linhares F, Puga MI, Rubio V, Pérez-Pérez J, Solano R, Leyva A, Paz-Ares J (2010) A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. PLoS Genet **6**: e1001102

Chandrika NNP, Sundaravelpandian K, Yu SM, Schmidt W (2013) ALFIN-LIKE 6 is involved in root hair elongation during phosphate deficiency in Arabidopsis. New Phytol **198**:709–720

Datta S, *et al.* (2015) Intensity of a pulse of RSL4 transcription factor synthesis determines Arabidopsis root hair cell size. Nat. Plants **1**:15138

Feller A, Machemer K, Braun EL, Grotewold E (2011) Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. Plant J. **66**:94-116.

Feng Y, Xu P, Li B, Wen X, An F, Gong Y, Xin Y, Zhu Z, Wang Y, Guo H. (2017). Ethylene promotes root haair growth through coordinated EIN3/EIL1 and RHD6/RSL1 activity in Arabidopsis. Proc Natl Acad Sci USA. doi/10.1073/pnas.1711723115

Hwang, Y. *et al.* (2017) Tracheophytes Contain Conserved Orthologs of a Basic Helix-loop-helix Transcription Factor to Modulate ROOT HAIR SPECIFIC Genes. Plant Cell doi:10.1105/tpc.16.00732

Lee RD-W and Cho H-T. (2013) Auxin, the organizer of the hormonal/environmental signals for root hair growth. Frontiers in Plant Sci **4**, 448. doi: 10.3389/fpls.2013.00448

Lee Y., M.C. Rubio, J. Alassimone, N. Geldner. (2013). A mechanism for localized lignin deposition in the endodermis. Cell **153**, 402-412.

Lin Q *et al.* (2015) GLABRA2 directly suppresses basic Helix-Loop-Helix transcription factor genes with diverse functions in root hair development. Plant Cell **27**:2894-906

Mangano S, *et al.* (2017). The molecular link between auxin and ROS-controlled root hair growth. Proc Natl Acad Sci USA. doi/10.1073/pnas.1701536114

Menand B, Yi K, Jouannic S, Hoffmann L, Ryan E, Linstead P, Schaefer DG, Dolan L (2007) An ancient mechanism controls the development of cells with a rooting function in land plants. Science **316**:1477-1480

Pires ND, *et al.* (2013) Recruitment and remodeling of an ancient gene regulatory network during land plant evolution. Proc. Natl. Acad. Sci. USA. **110**:9571-9576

Rymen B, *et al*. (2017) ABA Suppresses Root Hair Growth via the OBP4 Transcriptional Regulator. Plant Physiol. **173**(3):1710–1762

Song L, Yu H, Dong J, Che X, Jiao Y, Liu D (2016) The molecular mechanism of ethylene-mediated root hair development induced by phosphate starvation. PLoS Genet. 2016, 12: e1006194.

Velasquez, S.M. *et al.* (2016) Auxin and cellular elongation. Plant Physiol. **170**:1206-1215.

Yi, K. *et al.* (2010) A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. Nat. Gen. **42**:264-267

Zhang S. *et al.* (2016). Multiple phytohormones promote root hair elongation by regulating a similar set of genes in the root epidermis in Arabidopsis. J. Exp. Bot. **67**(22): 6363–6372

222 **Supplementary Information**

223 Material and Methods. Supplementary Figures S1-S2. Supplementary Tables S1-S2.

224

225

226 **Materials and Methods.**

227 **Plant Growth and mutant isolation**. *Arabidopsis thaliana* Columbia-0 (Col-0) was used as the wild

228 type (Wt) genotype in all experiments, unless stated otherwise. All mutants and transgenic lines

229 tested are in this ecotype. Seedlings were germinated on agar plates in a Percival incubator at 22°C

230 in a growth room with 16h light/8h dark cycles for 10 days at 140 µmol $m^{-2}s^{-1}$ light intensity. Plants

231 were transferred to soil for growth under the same conditions as previously described at 22°C. For

232 identification of T-DNA knockout lines, genomic DNA was extracted from rosette leaves.

233 Confirmation by PCR of a single and multiple T-DNA insertions in the target RBOH and PER genes

234 were performed using an insertion-specific LBb1 or LBb1.3 (for SALK lines) or Lb3 (for SAIL lines)

235 primer in addition to one gene-specific primer. To ensure gene disruptions, PCR was also run using

236 two gene-specific primers, expecting bands corresponding to fragments larger than in Wt. In this

237 way, we isolated homozygous lines (for all the genes mentioned above). Mutant list is detailed in

238 **Supplementary Table S1**.

239

240 **Growth media. RBOHs inhibition and auxin treatment.** Sterilized seeds were stored at 4°C in sterile

241 water were for 48 hours and then were germinated on agar plates containing modified Hoagland

242 solution contained 1 mM $Ca(NO_3)_2$, 50 µM $CaCl_2$, 0.25 mM $MgSO_4$, 50 µM Fe-NaEDTA, 1 mM KCl, 2

243 µM $MnSO_2 \cdot 2H_2O$, 0.5 µM $CuSO_4 \cdot 5H_2O$, 0.5 µM $Na_2MoO_4 \cdot 2H_2O$, 2 µM Zn $SO_4 \cdot 7H_2O$ and 2.5mM MES

244 containing low phosphate (5 µM $(NH_4)2HPO_4$). Media were adjusted to a pH of 5.7 and solidified

245 using 0.8% agar. After 5 days plants were transferred into to modified Hoagland solution with low

246 phosphate (5 µM $(NH_4)2HPO_4$) or high phosphate (5 mM $(NH_4)_2HPO_4$). Low and high phosphate

247 were combined with 100 nM of indole-3-acetic acid (IAA) or/and 15 µM of VAS2870 (VAS, for 3-

248 Benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo(4,5-d)pyrimidine). After 5 days (10 days old),

249 quantitative analysis of root hair phenotypes and total ROS measurements with H_2DCF-DA were

250 made.

251

252 **Root hair phenotype.** For quantitative analysis of root hair phenotypes in *rboh* mutants and Wt Col-

253 0, 200 fully elongated root hairs were measured (n roots= 30) from seedlings grown on vertical

254 plates for 10 days. Values are reported as the mean ±SD using the Image J 1.50b software.

255 Measurements were made on images were captured with an Olympus SZX7 Zoom microscope

256 equipped with a Q-Colors digital camera.

257

258 **H_2DCF-DA probe used to measure total ROS.** Arabidopsis seeds were grown in plates with sterile

259 agar 1% for 8 days in chamber at 22°C with continuous light. These seedlings were incubated with

10

260   2',7'-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) in darkness in a slide for 10 min 50 µM at
261   room temperature. Samples were observed with a confocal microscope equipped with 488 nm
262   argon laser and BA510IF filter sets. It was used the following configuration (10X objective, 0.30 NA;
263   4.7 laser intensity, 1.1 off set, 440 PMT for highest ROS levels and 480 PMT for ROS media and 3 of
264   gain. Images were taken scanning XZY with a 2 um between focal planes. Images were analyzed
265   using ImageJ. To measure ROS highest levels, a circular ROI (r=2.5) was taken in the zone of the root
266   hair with highest intensities. To measure ROS mean the total area of the root hair was taken.
267   Pharmacological treatments were carried out with a combination of the following reagents: 1-20
268   mM Pi, 100 nM IAA, and 15 µM of VAS2870. The sample was washed with a MS 0.5X solution and
269   the image acquisition was made with 10X objective and 400ms of exposure-time in an
270   epifluorescence microscope (Zeiss, Imager A2). To measure ROS levels a circular ROI (r=2.5) was
271   taken in the tip of the root hair. Values are reported as the mean ±SD using the Image J 1.50b
272   software.

273

274   **HyPer sensor to measure $_{cyt}$$H_2$$O_2$**. HyPer consists of a circularly permuted YFP (cpYFP) molecule
275   coupled to a regulatory domain of the *Escherichia coli* $H_2$$O_2$ sensor OxyR (1-4). When exposed to
276   $H_2$$O_2$, the excitation peak of cpYFP shifts from 420 to 500 nm, while the emission peak remains at
277   516 nm allowing it to be used as a ratiometric biosensor (Mangano et al. 2017). Ten day-old
278   *Arabidopsis* seedlings expressing the fluorescent HyPer biosensor were used. Root hairs were ratio
279   imaged with the Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss) using a 40X oil-
280   immersion, 1.2 numerical aperture. The HyPer biosensor was excited with both the 405 nm blue
281   diode laser and with the 488 nm argon laser. The emission (516 nm) was collected using a primary
282   dichroic mirror and the Meta-detector of the microscope. For time-lapse analysis, images were
283   collected every 3s. To measure ROS highest levels, a circular ROI (r=2.5) was taken in the root hair
284   tip for each image the time lapse. Treatments were made *in vivo* with 5mM Pi, 100 nM IAA, or 50
285   µM of VAS2870. Values are reported as the mean ±SD using the Image J 1.50b software.

286

287   **Quantitative reverse transcriptase PCR (qRT-PCR).** Total RNA was isolated from 10-d-old seedling
288   roots (40 for each line) using the RNAzol RT (MRC). cDNA was synthesized using M-MLV Reverse
289   Transcriptase (Promega). qRT-PCR analyses were performed using LightCycler480 SYBR Green I
290   Master-Roche. Gene-specific signals were normalized relatively to PP2A (AT1G69960;
291   serine/threonine protein phosphatase 2A) signals. Each qRT-PCR reaction was performed in
292   triplicate, and each experiment was repeated three times using independent preparations of RNA.
293   Primers used are as listed in **Supplementary Table S2**.

294 **Legend to Figure S1.**

295

296 **Figure S1. Auxin fails to overcome high-Pi root hair growth-repression in a low ROS-depleted**
297 **background (in *noxc* and *per44,73* mutants).**
298 Auxin fails to circumvent root hair growth repression imposed by increased levels of Pi in a ROS-
299 depleted *rbohc* mutant. Root hair length (mean $\pm$ SD) was measured in *rbohc* roots treated with
300 with 5mM inorganic Pi with or without 100nM IAA (indole 3-acetic acid) (on the left). Total ROS
301 levels generated by oxidation of H_2DCF-DA were measured at the root hair tip in different stages of
302 root hair development in the four different treatments (on the right).
303 Word count: 97

304

305 **Figure S2. Proposed models of auxin-RSL2/RSL4 regulation of ROS mediated polar root hair**
306 **growth in the absence and in presence of high-Pi**. Transcriptional responses under high-level of
307 auxin in the absence of Pi (A), in the presence of high Pi with very low-level of auxin (B), and in high
308 levels of Pi together with high levels of auxin (C) are shown.
309 A. The bHLH transcription factor RSL4 as well as RSL2 are both transcriptionally activated by high
310 levels of auxin (IAA) and its expression is directly regulated by several Auxin Response Factors
311 (ARFs; ARF5,7,8,19). Throughout RSL4 (and possibly RSL2), auxin activates the expression of two
312 RBOHs (RBOHC,J) and four PERs (PER1,44,60,73) that together regulate ROS homeostasis in the
313 apoplast (in combination with RBOHH). Based on Mangano et al. (2017) and the present work.
314 B. High level of Pi in a low auxin level environment would be sufficient to repress the RSL4/RSL2
315 transcriptional response. Pi repression act depleting auxin levels in the atricoblast (1) or repressing
316 by an unknown mechanism directly the expression of RSL4/RSL2 (2).
317 C. High levels of Pi in the presence of high levels of auxin are able to activate the expression of RSL2
318 and control ROS-homeostasis derived from RBOHs and PERs activities. Solid lines indicate
319 transcriptional activation or metabolite production. Reactive Oxygen Species (ROS) include hydroxyl
320 radical ($^{\bullet}OH$), superoxide ion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2).
321 Word count: 234

322

323 **Supplementary Table S1**. RSLs and NADPH oxidase C (RBOHC) mutant line used in this study. All are
324 in Col-0 background.
325

Name	Locus	Mutant name	Mutant code	References
RSLs				
RSL2	At3g33880	*rsl2-1*	SAIL line 514C04	Yi, K. et al. (2010)
RSL4	At1g27740	*rsl4-1*	GT_5_105706	Yi, K. et al. (2010)
RBOH				
RBOHC	At5g51060	*rbohc-1*	Salk_071801	Lee, Y. et al. (2013)

326
327

328 **Supplementary Table S2.** Primer List used for qPCR.
329

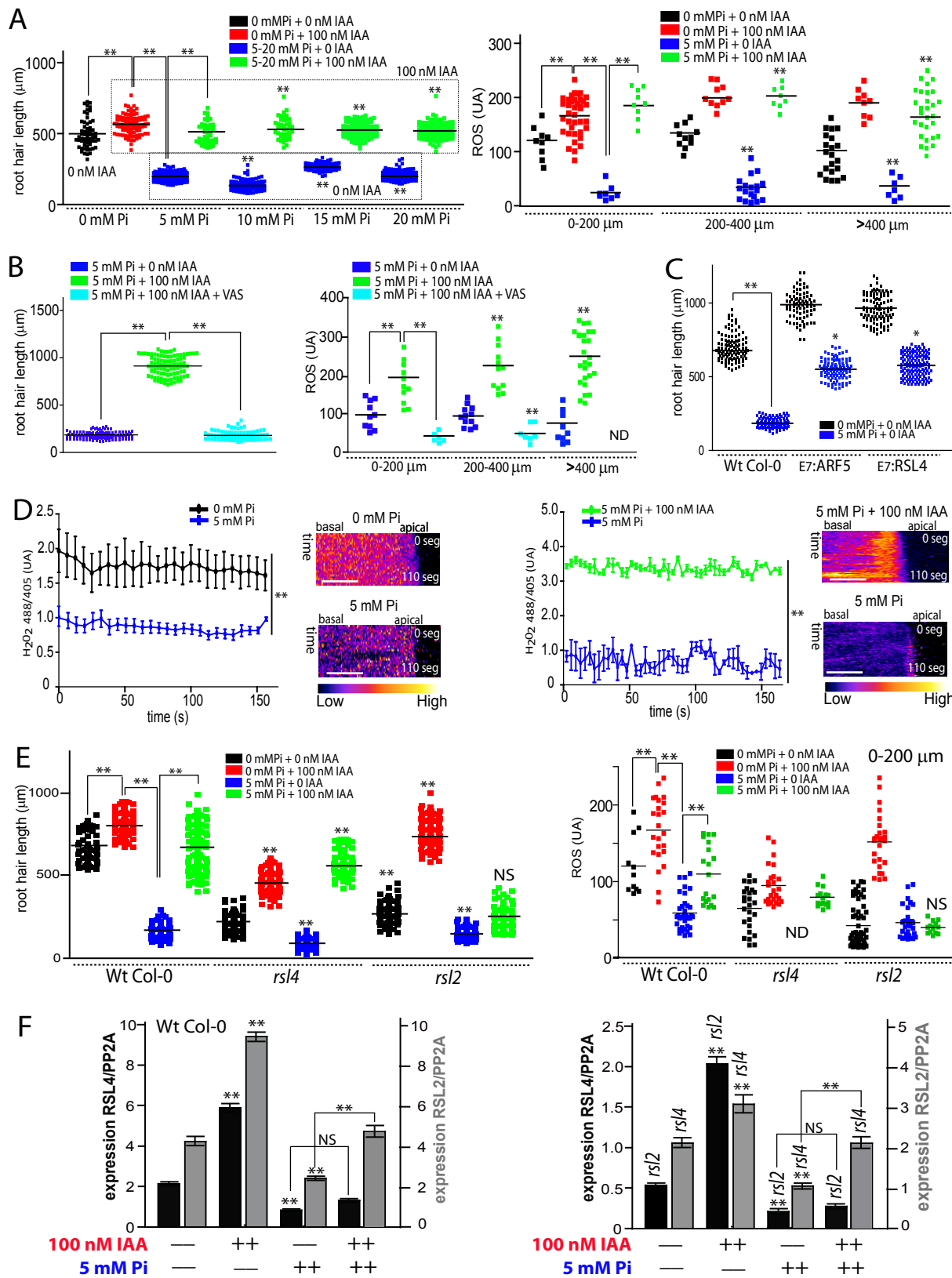| Gene	Position from start codon	Sequence	Direction
RSL2	896	CCCCAATGGAACAAAGGTC	Forward
At3g33880	1036	TCTCGGTGAGCTGAGACCAA	Reverse
RSL4	597	GTGCCAAACGGGACAAAAGT	Forward
At1g27740	735	TTGTGATGGAACCCCATGTC	Reverse
PP2A	1785	TAACGTGGCCAAAATGATGC	Forward
At1g69960	1845	GTTCTCCACAACCGCTTGGT	Reverse

330

Figure 1

Figure 1. Auxin overcomes high-Pi root hair growth-repression by enhancing RSL2 expression linked to ROS-homeostasis. A. Auxin circumvents root hair growth repression imposed by increased levels of Pi by enhancing ROS-production. Root hair length (mean ± SD) was measured in Wt Col-0 roots non-treated, treated with 100nM IAA (indole 3-acetic acid) or with 5mM inorganic Pi, or both treatment at the same time (on the left). Total ROS levels generated by oxidation of H_2DCF-DA were measured at the root hair tip in different stages of root hair development in the four different treatments (on the right). B. Auxin recovery of root hair growth is dependent on ROS-production. Root hairs treated with 100 nM IAA in the presence of high levels of Pi and VAS inhibitor fails to recover its normal growth (on the left). ROS-levels were down regulated in the treated root hairs with 100 nM IAA in the presence of high levels of Pi and VAS inhibitor (on the right). ND= root hairs not detected. C. High levels of ARF5 and RSL4 expression in root hairs cells (EXPANSIN 7 promoter, E7) are able to partially overcome the growth repression imposed by high levels of Pi on cell elongation. Comparisons are made between Wt Col-0 and _E7_:ARF5 or _E7_:RSL4 lines under high levels of Pi. D. Auxin trigger changes in _cyt_H_2O_2 levels detected with HyPer. _cyt_H_2O_2 levels Wt Col-0 root hairs expressing HyPer sensor treated with 5 mM Pi and 100 nM IAA. _cyt_H_2O_2 levels are based on the ratio 488/405 nm of HyPer biosensor at the root hair tip over 200 s. On the right, selected kymographs resulting of this analysis only for root hairs of >200 μm in length. Scale bar = 5 μm. E. RSL2 but not RSL4 mediates the auxin recovery response in the presence of high Pi-levels. Root hair length (mean ± SD) was measured in Wt Col-0, _rsl4_ and _rsl2_ roots non-treated, treated with 100nM IAA (indole 3-acetic acid) or with 5mM inorganic Pi, or both treatments at the same time. ROS-levels were partially recovered in Wt but not in _rsl2_ when treated with 100 nM IAA in the presence of high levels of Pi. No ROS was detected in _rsl4_ under high levels of Pi. Only root hairs <200 μm in length were analyzed. F. Auxin under high Pi-levels triggers the expression of both, RSL4 and RSL2. Levels of RSL4 and RSL2 expression in Wt Col-0 roots non-treated treated with 100nM IAA (indole 3-acetic acid) or with 5mM inorganic Pi, or both treatments at the same time (on the left). Levels of RSL4 and RSL2 expression in _rsl2_ and _rsl4_ respectively non-treated treated with 100nM IAA (indole 3-acetic acid) or with 5mM inorganic Pi, or both treatments at the same time (on the left). Each qRT-PCR reaction was performed in triplicate, and each experiment was repeated three times using independent preparations of RNA. Comparisons are made in the same genetic background between levels of gene expression (of RSL2 or RSL4) in non-treated samples (no IAA/no Pi) versus treated with IAA or Pi. In addition, Pi-treated samples are compared only to Pi+IAA-treated roots. In Figs. 1A-E, _P_-value of one-way ANOVA, (**) P<0.001, (*) P<0.01. NS= not significant different. In A-E Error bars indicate ±SD from 3 biological replicates.
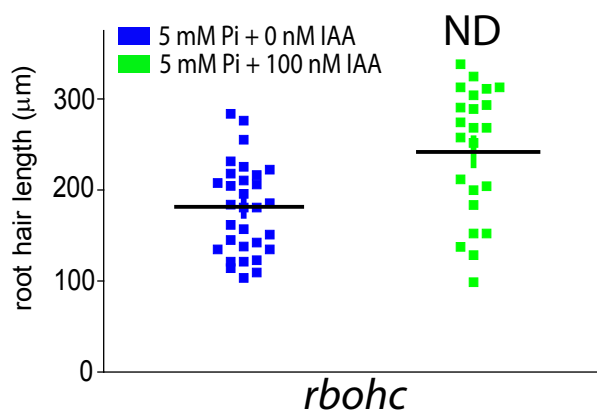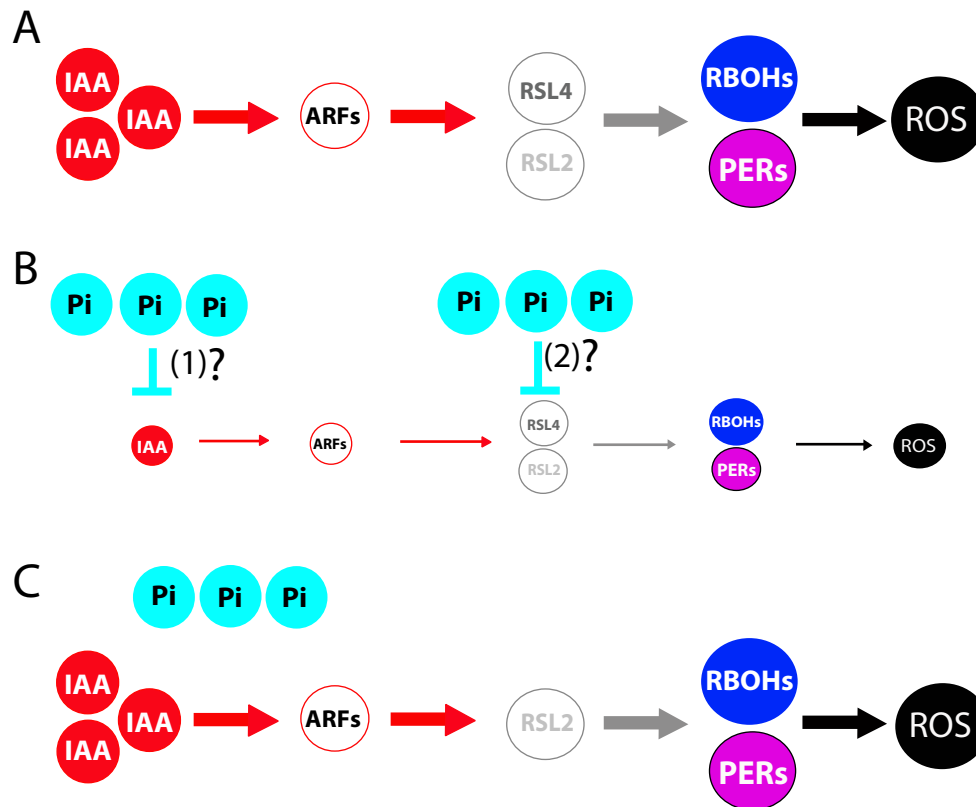
Figure S1

Figure S2

Figure S2. Proposed models of auxin-RSL2/RSL4 regulation of ROS mediated polar root hair growth in the absence and in presence of high-Pi. Transcriptional responses under high-level of auxin in the absence of Pi (A), in the presence of high Pi with very low-level of auxin (B), and in high levels of Pi together with high levels of auxin (C) are shown. A. The bHLH transcription factor RSL4 as well as RSL2 are both transcriptionally activated by high levels of auxin (IAA) and its expression is directly regulated by several Auxin Response Factors (ARFs; ARF5,7,8,19). Throughout RSL4 (and possibly RSL2), auxin activates the expression of two RBOHs (RBOHC,J) and four PERs (PER1,44,60,73) that together regulate ROS homeostasis in the apoplast (in combination with RBOHH). Based on Mangano et al. (2017) and the present work. B. High level of Pi in a low level environment of auxin would be sufficient to repress the RSL4/RSL2 transcriptional response. Pi repression may act at the level of depleting auxin levels in the atricoblast (1) or by repressing by unknown mechanism directly the expression of RSL4/RSL2 (2). C. High levels of Pi in the presence of high levels of auxin are able to activate the expression of RSL2 and control ROS-homeostasis derived from RBOHs and PERs activities. Solid lines indicate transcriptional activation or metabolite production. Reactive Oxygen Species (ROS) include hydroxyl radical ($^{\bullet}$OH), superoxide ion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2).