Molecular link between auxin and ROS-mediated polar growth

Silvina Mangano^{a,b,c,1}, Silvina Paola Denita-Juarez^{a,b,c,1}, Hee-Seung Choi^{d,1}, Eliana Marzol^{a,b,c,1}, Youra Hwang^d, Philippe Ranocha^e, Silvia Melina Velasquez^{a,b,c,2}, Cecilia Borassi^{a,b,c}, María Laura Barberini^f, Ariel Alejandro Aptekmann^g, Jorge Prometeo Muschietti^{f,h}, Alejandro Daniel Nadra^g, Christophe Dunand^e, Hyung-Taeg Cho^{d,3}, and José Manuel Estevez^{a,b,c,3}

^aFundación Instituto Leloir, Buenos Aires C1405BWE, Argentina; ^bIIBBA-CONICET, Buenos Aires C1405BWE, Argentina; ^cInstituto de Fisiología, Biología Molecular y Neurociencias, IFIByNE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Buenos Aires C1428EGA, Argentina; ^dDepartment of Biological Sciences, Seoul National University, Seoul 151-742, Korea; ^eLaboratoire de Recherche en Sciences Végétales, Université de Toulouse, Université Paul Sabatier, UMR 5546, CNRS, F-31326 Castanet-Tolosan, France; ^fInstituto de Investigaciones en Ingeniería Genética y Biología Molecular, Dr. Héctor Torres, INGEBI-CONICET, Buenos Aires 1428, Argentina; ^gDepartamento de Química Biológica, IQUIBICEN-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Buenos Aires C1428EGA, Argentina; and ^hDepartamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Buenos Aires C1428EGA, Argentina

Edited by Natasha V. Raikhel, Center for Plant Cell Biology, Riverside, CA, and approved April 10, 2017 (received for review March 7, 2017)

Root hair polar growth is endogenously controlled by auxin and sustained by oscillating levels of reactive oxygen species (ROS). These cells extend several hundred-fold their original size toward signals important for plant survival. Although their final cell size is of fundamental importance, the molecular mechanisms that control it remain largely unknown. Here we show that ROS production is controlled by the transcription factor RSL4, which in turn is transcriptionally regulated by auxin through several auxin response factors (ARFs). In this manner, auxin controls ROS-mediated polar growth by activating RSL4, which then up-regulates the expression of genes encoding NADPH oxidases (also known as RESPIRATORY BURST OXIDASE HOMOLOG proteins) and class III peroxidases, which catalyze ROS production. Chemical or genetic interference with ROS balance or peroxidase activity affects root hair final cell size. Overall, our findings establish a molecular link between auxin and ROS-mediated polar root hair growth.

auxin | root hair growth | RSL4 | ROS | peroxidases

In *Arabidopsis thaliana*, root hair cells and non-hair cell layers differentiate from the epidermis in the meristematic zone of the root. Once root hair cell fate has been determined, root hairs protrude from the cell surface and represent up to 50% of the surface root area; this process is crucial for nutrient uptake and water absorption. Root hair growth is controlled by the interplay of several proteins, including the transcription factor (TF) ROOT HAIR DEFECTIVE 6 (RHD6), a class I RSL protein belonging to the basic helix-loop-helix (bHLH) family (1). The bHLH transcription factor RSL4 (ROOT HAIR DEFECTIVE SIX-LIKE 4), which defines the final root hair length based on its level of expression (2), is a central growth regulator acting downstream of RHD6. Other TFs also contribute to the regulation of root hair growth (3, 4). Root hair cell elongation is modulated by a wide range of both environmental signals (5, 6) and endogenous hormones, such as ethylene and auxin (2, 5, 7). However, the underlying mechanisms are unknown. The transcriptional auxin response is mediated by auxin binding to receptors of the TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) family and its coreceptor AUXIN/INDOLE 3-ACETIC ACID (Aux/IAA). This triggers the proteasome-dependent degradation of Aux/IAA, which results in the release of the auxin response factors (ARFs). The released ARFs bind to *cis*-auxin response elements (Aux-REs) in the promoters of early auxin response genes to trigger downstream responses (8). Gain-of-function mutants for several Aux/IAAs exhibit reduced root hair growth (9). On the other hand, a high degree of genetic redundancy is expected in root hairs because several ARFs are highly expressed in trichoblast cells [e.g., ARF7 and

ARF19 are the two most abundant ARFs (10)]. In agreement, the *arf7arf19* double mutant did not show a reduction in root cell elongation (11), indicating that there are other ARFs acting downstream of auxin in growing root hair cells. Auxin needs to be sensed in situ in hair cells to trigger cell expansion, although no molecular connection has been proposed yet between ARFs and downstream TFs that control growth (SI Appendix, Fig. S1). Downstream auxin signaling is sustained by an oscillatory feedback loop composed of two main components, calcium ions (Ca^{2+}) and reactive oxygen species (ROS). High levels of cytoplasmic Ca^{2+} ($\text{Ca}^{2+}_{\text{cyt}}$) trigger ROS production by RBOHs (RESPIRATORY BURST OXIDASE HOMOLOG proteins), and high levels of Ca^{2+} activate unknown Ca^{2+} -permeable channels that promote Ca^{2+} influx into the cytoplasm (12, 13). Plant RBOHs are plasma membrane-localized NADPH oxidases that produce apoplastic superoxide ion (O_2^-), which is mostly converted chemically or enzymatically into hydrogen peroxide (H_2O_2). *Arabidopsis* RBOHs

Significance

Tip-growing root hairs are an excellent model system for deciphering the molecular mechanism underlying reactive oxygen species (ROS)-mediated cell elongation. Root hairs are able to expand in response to external signals, increasing several hundred-fold their original size, which is important for survival of the plant. Although their final cell size is of fundamental importance, the molecular mechanisms that control it remain largely unknown. In this study, we propose a molecular mechanism that links the auxin-auxin response factors module to activation of RSL4, which directly targets genes encoding ROS-producing enzymes such as NADPH oxidases (or RESPIRATORY BURST OXIDASE HOMOLOG proteins) and secreted type III peroxidases. Activation of these genes impacts on apoplastic ROS homeostasis, thereby stimulating root hair cell elongation.

Author contributions: S.M., S.P.D.-J., H.-S.C., E.M., S.M.V., H.-T.C., and J.M.E. designed research; S.M., S.P.D.-J., H.-S.C., E.M., Y.H., P.R., S.M.V., C.B., M.L.B., C.D., and H.-T.C. performed research; S.M., S.P.D.-J., H.-S.C., E.M., Y.H., P.R., H.-T.C., and J.M.E. contributed new reagents/analytic tools; E.M., Y.H., P.R., C.B., M.L.B., A.A.A., J.P.M., A.D.N., C.D., H.-T.C., and J.M.E. analyzed data; and H.-T.C. and J.M.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹S.M., S.P.D.-J., H.-S.C., and E.M. contributed equally to this work.

²Present address: Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, 1190 Vienna, Austria.

³To whom correspondence may be addressed. Email: htcho@snu.ac.kr or jestevez@leloir.org.ar.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1701536114/-DCSupplemental.

(AtRBOHA-J) are associated with diverse ROS-related growth responses (14–16); for instance, RBOHC promotes root hair budding (17, 18), and RBOHH_J are required for proper pollen tube growth (19). In addition, the pool of apo-ROS can be modulated by the activity of class III peroxidases (PERs) (20). The mechanism that links auxin to ROS-mediated cell growth is unclear (*SI Appendix*, Fig. S1). Here we propose a molecular mechanism in which endogenous auxin activates several ARFs to up-regulate the expression of *RSL4*, which controls ROS-mediated polar root hair growth by promoting the expression and activity of RBOH and PER proteins. On the other hand, we were not able to detect an auxin-mediated nontranscriptional fast response to trigger ROS production in root hair cells.

Results and Discussion

Several ARFs Mediate *RSL4* Activation, Linking Auxin-Dependent Polar Growth to ROS Production in Root Hair Cells. To establish whether ROS production is linked to auxin-dependent polar growth, we analyzed root hair growth and ROS levels in the presence of exogenously supplied auxin (100 nM IAA). Auxin enhances root hair growth (Fig. 1*A* and *SI Appendix*, Fig. S2*A*) and triggers a slightly higher ROS level in root hairs longer than 300 μ m (*SI Appendix*, Fig. S2*B*). Next, we monitored cytoplasmic H₂O₂ (cytH₂O₂) levels using a genetically encoded YFP-based H₂O₂ sensor, named HyPer, which reacts with H₂O₂ when external apoH₂O₂ is applied in the growing tip (*SI Appendix*, Fig. S3*A*). H₂O₂ levels in auxin-treated roots maintain a moderate HyPer signal in root hairs longer than 700 μ m, whereas there is no root hair development in nontreated ones that reach these cell lengths (*SI Appendix*, Fig. S3*B*). Hence, the maintenance of cytROS levels is a reliable response to auxin in tip-growing root hairs. Auxin triggers polar root hair growth via an unknown mechanism involving *RSL4* activation (5). First, we analyzed whether both *RSL2* and *RSL4* directly regulate ROS production to trigger root hair growth. The *rsl2-1 rsl4-1* double mutant showed highly reduced ROS levels, whereas slightly higher ROS levels were detected in the *RSL4*-overexpressing line (*RSL4*^{OE}) than in the wild type (WT) during late stages of root hair development (≥ 400 μ m in length) (Fig. 1*B* and *C*). Therefore, both active *RSL2* and *RSL4* stimulate ROS production (Fig. 1*C* and *SI Appendix*, Fig. S2*D*). We then tested whether auxin triggers ROS production in an *RSL2*/*RSL4*-dependent manner. Auxin partially stimulated root hair growth and ROS production in each of the *rsl4-1* and *rsl2-1* null mutants but failed in the *rsl2-1 rsl4-1* double mutant (Fig. 1*A* and *B* and *SI Appendix*, Fig. S2*C* and *D*). Mutants for other reported TFs involved in root hair growth, such as *manyb* (3) and *hdg11* (4), were treated with auxin, but normal responses were observed, suggesting that these TFs are not involved in auxin activation (*SI Appendix*, Fig. S4). In addition, the *RSL4*^{OE} line was less sensitive to the RBOH inhibitor VAS2870 (VAS) with respect to cell growth-linked ROS production (*SI Appendix*, Fig. S5). Overall, these findings highlight the key role of both *RSL2* and *RSL4*, although *RSL4* has a more protagonist role as a main transcriptional activator of the ROS-related auxin response in root hairs.

Next, we tested whether ARFs regulate root hair growth by up-regulating *RSL4* expression and ROS production. Several ARFs, such as ARF5, ARF7, ARF8, and ARF19, are expressed in the meristematic and elongation zones of the root as well as in trichoblast cells and developing root hairs (*SI Appendix*, Fig. S6*A* and *B*). Based on the lack of phenotype for the *arf7 arf19* mutant (11), all data suggest a high degree of functional redundancy between these ARFs possibly involved in root hair growth. To overcome this, we then generated lines overexpressing these ARFs under the control of the strong root hair-specific promoter EXP7 (*p₇:ARF*; *SI Appendix*, Fig. S6*C*). Root hair growth was enhanced in three overexpression lines tested, especially in the ARF5 line (Fig. 1*D* and *SI Appendix*, Fig. S6*C*). Furthermore, *RSL4* expression was elevated

Fig. 1. ARF-mediated *RSL4* activation links auxin-dependent polar root hair growth to ROS production. (A) Root hair length (mean $\pm$ SD) in WT Col-0, *rsl4-1*, and *rsl2-1 rsl4-1* treated with 100 nM IAA. (B) Effect of auxin (100 nM IAA) mediated by *RSL4* on ROS production. ROS signal analysis with IAA treatment of WT Col-0 and the *rsl4-1* mutant in the early stages of root hair development. AU, fluorescent arbitrary units. (C) ROS in WT Col-0 and the *RSL4* overexpressor in later stages of root hair growth. (D) Effect of EXP7 (*EXPANSIN 7*)-driven ARF5 (*p₇:ARF5*^{OE}), ARF7 (*p₇:ARF7*^{OE}), and ARF8 (*p₇:ARF8*^{OE}) overexpression on polar root hair growth (black bars) and *RSL4* expression (orange bars) (mean $\pm$ SD). (E) ROS in EXP7-GFP/WT Col-0 (*p₇:GFP* as control) and the EXP7-driven ARF5,7,8 (*p₇:ARF5,7,8*^{OE}) overexpressor in the later stages of root hair development. (F) ChIP analysis showing ARF5 binding to auxin response elements on the *RSL4* promoter region. The *RSL4* promoter region (*pRSL4*) and the relative positions of Aux-REs (red bars) and ChIP-PCR regions (lines 1 to 9) are indicated. (Bottom) The enrichment fold of ARF5-GFP in ChIP-PCR of each region shown. Cont, control, empty vector line; ARF5, *pMDC7:ARF5*-GFP, dexamethasone-inducible ARF5-GFP line. The values are relative to each Cont value and significantly different (***P* < 0.001; **P* < 0.005; *t* test) from each Cont value. (G) Proposed events from the IAA signal mediated by ARF5 activation of *RSL4* expression to ROS-linked root hair (RH) growth. (A–E) One-way ANOVA; ***P* < 0.001, **P* < 0.01. ND, not detected; NS, not significantly different. Error bars indicate $\pm$ SD of biological replicates.

(Fig. 1*D*) and high ROS levels were maintained in the root hair cells of these lines during late developmental stages (Fig. 1*E*). Given that the region upstream of *RSL4* contains eight ARF response elements (Fig. 1*F* and *SI Appendix*, Table S2), we speculated that these ARFs would be able to bind to the *RSL4* promoter and trigger its expression. To test this possibility, we carried out chromatin immunoprecipitation (ChIP) analyses (Fig. 1*F*) using an estradiol-inducible version of ARF5-GFP because it was the most efficient ARF tested to up-regulate *RSL4* expression. ChIP of ARF5 showed that the fold-enrichment levels are significantly higher in the Aux-RE regions than in the control (Fig. 1*F*). These data indicate that ARF5 binds to Aux-REs in the *RSL4* promoter region and positively regulates its expression. It is highly possible that ARF7 and ARF8 also bind and regulate *RSL4* expression. Previously, it was shown that the *Aux/IAA14 slr1-1* resistant mutant lacked root hairs (9), suggesting a strong inhibition of these ARFs. To test whether a single ARF is able to overcome this inhibition, we selected ARF19 because it is one of the ARFs most highly expressed in root hair cells (10). High levels of ARF19 (*ARF19*^{OE}; overexpression) were transgenically expressed in the *Aux/IAA14 slr1-1* root hair-less background. *ARF19*^{OE} was able to completely rescue the *slr1-1* root hair phenotype and normalize ROS levels (*SI Appendix*, Fig. S7*A* and

B). As expected, ARF19 nuclear expression is highly responsive to auxin and is localized in root epidermal cells close to the root hair development zone (SI Appendix, Figs. S6B and S7C). Together, these results and previous reports (9–11) suggest that high auxin levels in trichoblast cells would release several ARFs (e.g., ARF5, ARF7, ARF8, and ARF19) from its repressors Aux/IAAs, to directly control RSL4 expression (and possibly RSL2), triggering ROS-mediated root hair elongation (Fig. 1F). Further experiments are required to establish how these ARFs are regulated to act in a coherent manner to trigger the transcriptional response during cell growth.

RSL4-Regulated *RBOHC, J* as Well as *RBOHH* Drive ROS-Mediated Root Hair Growth. Besides *RBOHC*, two other *RBOH* groups exist: One group clusters *RBOHC* and is composed of *RBOHE, F* (SI Appendix, Fig. S8A), and the other was previously implicated in polar growth of pollen tubes (19) and is composed of *RBOHH, J*. To identify which of these *RBOH*s also contribute to ROS-linked tip growth, we isolated *rbob* mutants (SI Appendix, Fig. S8B and Table S1) and screened them for abnormal cell expansion linked to deficient ROS production (Fig. 2A and B). Only *rbobc-1* and *c-2* showed short root hairs, whereas *rbobh-1* and *h-3* developed slightly less elongated cells (SI Appendix, Fig. S8C and D). When double *rbob* mutants were analyzed, only *rbobh, j* and mutants containing *rbobc* showed a clear root hair growth reduction of up to 40% (Fig. 2A). In agreement with this, *rbobc* mutants showed highly reduced ROS levels, whereas double *rbobh, j* mutants presented moderate to low ROS levels (Fig. 2B and SI Appendix, Fig. S8E and F). We then used the HyPer H_2O_2 sensor to test whether the *RBOH* inhibitor VAS could limit the level of $cytH_2O_2$ in the growing root hair tip. The level of $cytH_2O_2$ was significantly diminished after VAS treatment (Fig. 2C), and $cytH_2O_2$ signatures were drastically modified in *rbobc-1* root hairs (Fig. 2D). Exogenous H_2O_2 treatment failed to rescue the *noxc-1* root hair growth defect (SI Appendix, Fig. S9A), implying that a complex and fine-tuned ROS regulation would be required to trigger growth. Together, these data suggest that although *RBOHC* is the main *RBOH* involved in ROS-mediated root hair growth, as reported previously (12, 17), *RBOHH, J* are also important ROS-producing enzymes in this process.

Based on an analysis of RSL4-regulated genes (5), a putative RSL4 response element (RSL4-RE) with the sequence $TN_6CA[CT]G[TA]$ was identified that is highly similar to the root hair *cis*-element (RHE) $TN_5-6CACG[TA]$. The promoter regions of the *RBOHC*, *H*, and *J* genes include several RHEs, suggesting that these three *RBOH*s could be direct targets of the RSL4 TF (SI Appendix, Table S2). Using ChIP analysis, we examined whether RSL4-GFP could bind to the promoter regions of *RBOHC, H, J* in vivo. Except for the *RBOHH* gene, RSL4 bound to at least one RHE region in the *RBOHC* and *RBOHJ* promoters (Fig. 2E). This result suggests that RSL4 directly regulates these two ROS-related genes to generate the ROS required for tip growth.

Why are three *RBOH* proteins needed to trigger polar growth in a single cell? The simplest hypothesis is that these *RBOH*s act sequentially. Low levels of ROS were detected when the root hair first emerged from the root, but the ROS signal was up to twofold greater in slightly longer WT Col-0 root hairs (of 200 to 250 μm in length) (SI Appendix, Fig. S9D and E). At a comparable stage, root hairs of the *rbobc-1* mutant had very low levels of $cytROS$ and those of the *rbobh, j* double mutant had only slightly lower ROS levels than did those of WT Col-0. In later growth stages, $cytROS$ levels were drastically reduced in the double *rbobh, j* mutant. This suggests that *RBOHC* is the first *RBOH* to produce ROS, and that *RBOHC* functionally overlaps with *RBOHH, J*. Finally, *RBOHH, J* produced ROS that was linked to root hair tip elongation at later developmental stages (>200 μm in length) (SI Appendix, Fig. S9E). Next, we investigated whether ROS is required for root hair initiation by subjecting WT Col-0 roots to

Fig. 2. RSL4-regulated *RBOHC, J*, as well as *H* drive ROS-mediated root hair growth. (A, Right) Root hair phenotype in WT Col-0 and *rbob* mutants. (Scale bars, 600 μm .) (Left) Root hair length (mean $\pm$ SD; $n = 30$ roots) in *rbob* mutants. (B) Total ROS generated by oxidation of $H_2DCF-DA$ in early stages of root hair development in *rbob* mutants. (Right) Total ROS $H_2DCF-DA$ staining in roots of WT Col-0 and *rbob* mutants. (Scale bars, 600 μm .) (C) $cytH_2O_2$ levels in VAS-treated WT Col-0 root hairs expressing the HyPer sensor. $cytH_2O_2$ levels are based on the ratio 488/405 nm of HyPer biosensor at the root hair tip over 600 s. (Right) Selected kymographs resulting from this analysis only for root hairs of >200 μm in length. (Scale bars, 5 μm .) (D) $cytH_2O_2$ average levels in *rbobc-1* and WT Col-0 root hairs expressing HyPer. Average $cytH_2O_2$ levels are based on the 488/405 nm ratio of HyPer at the root hair tip in root hairs of 0 to 200 μm in length. (Right) Selected kymographs. (Scale bars, 5 μm .) (E) Promoter regions of ROS-related genes *RBOHC*, *H*, and *J* as targets of the RSL4 response element (red bars) and ChIP-PCR regions (lines 1 to 4). Neighboring gene exons are indicated as a gray box. Enrichment fold of RSL4-GFP in ChIP-PCR for each region shown. RSL4, pRSL4:RSL4-GFP. Error bars indicate $\pm$ SD of biological replicates ($n = 2$). The values are relative to each Cont value. Significantly different (** $P < 0.01$, * $P < 0.05$; t test) from the Cont value. (F) Proposed events from RSL4 activation of *RBOHC, J* to ROS-mediated polar root hair growth. (A, B, and D) One-way ANOVA; ** $P < 0.001$, * $P < 0.01$. Error bars indicate $\pm$ SD of biological replicates.

VAS. The concentration of VAS needed to inhibit root hair growth by half (IC_{50}) was $\sim 7.5 \mu M$ (SI Appendix, Fig. S9B), which is similar to ROS VAS IC_{50} ($\sim 7.0 \mu M$) (SI Appendix, Fig. S9C). The *rbobc-1* mutant (12) showed very short root hairs up to 150 μm in length (SI Appendix, Fig. S9B) but still produced $\sim 30\%$ of ROS compared with WT root hairs (Fig. 2D and SI Appendix, Fig. S9C), indicating the presence of residual ROS production (17). Because ROS homeostasis could be modified by the activity of PER, we treated WT and *rbobc-1* mutant roots with the PER

inhibitor SHAM (salicylhydroxamic acid) (21) at concentrations of up to 100 μ M. SHAM treatment resulted in a significant reduction in total ROS in *rboh1* in comparison with nontreated *rboh1*, although root hair initiation was not abolished (SI Appendix, Fig. S10). In an analysis using HyPer, 100 μ M SHAM completely abolished the H_2O_2 signal in the whole root (SI Appendix, Fig. S11), confirming that PERs also contribute to ROS production. This implies that, at least in *A. thaliana*, initial development of the root hair tip is not affected by highly reduced ROS levels.

RSL4-Regulated PERs Affect ROS Homeostasis and Polar Root Hair Growth. Next, coexpression analysis revealed that *RSL4* is highly coregulated at the transcriptional level with several *PER* genes, most of which exhibit root hair expression (SI Appendix, Fig. S12 A and B) (5). We then analyzed the corresponding T-DNA *per* mutants (SI Appendix, Fig. S12C and Table S1). The single mutant *per1-2* and double mutant *per44-2,73-3* (all null mutants except for *per1-2* knockdown; SI Appendix, Fig. S12D) showed mild phenotypes (short root hairs), with up to ~30% reductions in ROS levels (Fig. 3 A and B). This suggests that there is genetic redundancy between these PERs in the regulation of ROS homeostasis. The levels of $c_{yt}H_2O_2$, as visualized with HyPer, were reduced in root hairs treated with 75 μ M SHAM (Fig. 3C), and this result was confirmed using a 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF -DA) probe (SI Appendix, Fig. S10B). In addition, peroxidase activity was significantly lower in SHAM-treated roots as well as in *per* mutants and higher in *PER44* overexpressor (*PER44*^{OE}) than in WT nontreated ones (Fig. 3D), suggesting a direct link between peroxidase activity and ROS homeostasis. To explore the transcriptional regulation of these *PERs* in ROS-mediated root hair growth, we analyzed the regulatory region of the genes encoding these *PERs*. We identified several putative RHE motifs (SI Appendix, Table S3), suggesting that these genes could be direct targets of *RSL4*, as suggested by the coexpression analysis (SI Appendix, Fig. S12A). ChIP assays revealed that *RSL4* binds to the RHE of four *PERs*, *PER1*, 44, 60, and 73 (Fig. 3E). In agreement with this, much higher and lower levels of *PER1* and *PER73* were detected in *RSL4*^{OE}/WT and WT/*rsl4-1* lines, respectively (5), suggesting that *RSL4* promotes the expression of at least these two *PERs*. At last, higher peroxidase activity was found in *p7:ARF5* and *p7:ARF8* lines (where *RSL4* expression is up-regulated; Fig. 1D) and much lower activity was detected in *rsl4-1*, which lacks *RSL4* (Fig. 3F), confirming that *RSL4* not only directs *PER* expression at the transcriptional level but also impacts on the *PER* activity (Fig. 3G).

We then examined whether auxin treatment could compensate for the lack of RBOH and *PER* proteins (Fig. 4 A and B). Auxin treatment partially rescued the reduced length and ROS levels of the *rboh1* mutant, resulting in ~30% increases in both, and almost fully rescued the defects in the *rboh1,j* and *per44-2,73-3* mutants (Fig. 4 A and B). When 15 μ M VAS was added along with the auxin (IAA), the *rboh1* mutant exhibited intermediate root hair growth and partial recovery of ROS levels (SI Appendix, Fig. S13 A and B), suggesting other sources of ROS. When SHAM was added in addition to VAS (SHAM+VAS) in the presence of auxin, a low recovery of root hair growth and ROS levels was detected in *rboh1* and WT root hairs (SI Appendix, Fig. S13 C and D). Together, these findings confirm that auxin requires both RBOH-derived ROS and ROS produced by *PER* to modulate polar growth (SI Appendix, Fig. S13E). The small although detectable recovery in ROS and cell growth after auxin treatment in root hair cells (in *rboh1* as well as in WT) in the presence of both inhibitors, VAS and SHAM (SI Appendix, Fig. S13 C and D), would possibly suggest the existence of an unknown source of ROS coming from other apoplastic ROS-producing enzymes (e.g., oxalate oxidase, diamine oxidase, lipoxigenases, etc.) also regulated

Fig. 3. RSL4-regulated *PERs* affect ROS homeostasis and polar root hair growth. (A) Root hair length (mean $\pm$ SD; $n = 30$ roots) in the single mutant *per1-2* and double mutant *per44-2,73-3*. (B) ROS in root hairs of the single mutant *per1-2* and double mutant *per44-2,73-3*. (Right) ROS H_2DCF -DA staining in roots of WT Col-0 and *per* mutants. (Scale bars, 600 μ m.) (C) Average $c_{yt}H_2O_2$ levels in SHAM-treated and nontreated WT Col-0 root hairs expressing the HyPer sensor ($n = 10$). Average $c_{yt}H_2O_2$ levels are based on the 488/405 nm ratio of HyPer at the root hair tip over 100 s. One-way ANOVA; ** $P < 0.001$. (Right) Selected kymographs. (Scale bars, 5 μ m.) (D) Peroxidase activity in the roots of WT, *per1-2*, *per44-2*, and *per73-3* simple mutants, *per44-2,73-3* double mutant, and wild-type root extracts with increasing concentrations of SHAM. Enzyme activity (expressed as n katal/mg protein) was determined by a guaiacol oxidation-based assay. Values are the mean of three replicates $\pm$ SD. (E) Promoter regions of ROS-related genes *PER1*, 44, 60, 73 showing the *RSL4* response elements (red bars) and ChIP-PCR regions (lines 1 to 4). Positions are relative to the start codon. Neighboring gene exons are indicated as gray boxes. Enrichment fold of *RSL4*-GFP in ChIP-PCR of each region is shown. *RSL4*, pRSL4:GFP. Error bars indicate $\pm$ SD of biological replicates ($n = 2$). The values are relative to each WT value and significantly different (** $P < 0.01$, * $P < 0.05$; t test). (F) Peroxidase activity in the roots of WT, EXP7-driven GFP (as control), *ARF5* (*p7:ARF5*^{OE}), *ARF7* (*p7:ARF7*^{OE}), and *ARF8* (*p7:ARF8*^{OE}) as well as in the *rsl4-1* mutant. Enzyme activity (expressed as n katal/mg protein) was determined by a guaiacol oxidation-based assay. Values are the mean of three replicates $\pm$ SD. (G) Proposed events from *RSL4* activation of *PER1*, 44, 60, 73 to ROS-linked root hair growth. (A, B, D, and F) One-way ANOVA, ** $P < 0.001$, * $P < 0.01$. Error bars indicate $\pm$ SD of biological replicates.

by auxin-*RSL4* (and possibly *RSL2*) (SI Appendix, Fig. S13E), which will require further investigation.

Finally, we tested whether auxin is able to trigger a rapid and nontranscriptional response impacting on ROS production in the root hair tip (SI Appendix, Fig. S14). We applied IAA in growing root hairs that express the HyPer sensor, and no significant changes were detected before or after hormone treatment (SI Appendix, Fig. S14 A and B). A similar result was obtained when ROS levels were measured with the dye H_2DCF -DA (SI Appendix, Fig. S14C). Overall, our results suggest that the main auxin downstream targets are *RSL4* (and possibly *RSL2*) and the transcriptional activation of genes related to ROS production and involved in polar growth (SI Appendix, Fig. S13E). We cannot exclude other direct nontranscriptionally activated targets involved in the auxin response in root hair cells, but we fail to detect changes in H_2O_2 /ROS upon auxin stimulation in our experimental conditions.

Conclusions

A root hair is a single-cell protrusion able to extend in response to external signals, increasing several hundred-fold its original size, which is important for survival of the plant. Final root hair size has vital physiological implications, determining the surface area/volume ratio of the whole roots exposed to nutrient pools, thereby likely impacting nutrient uptake rates. Although the final hair size is of fundamental importance, the molecular mechanisms that

growing root hairs (17) directly regulate the cycles of cytH_2O_2 influx. Once H_2O_2 molecules are transported into the cytoplasm, they trigger downstream responses linked to polar growth (12, 18).

Materials and Methods

Root Hair Phenotype. For quantitative analysis of root hair phenotypes in *rbho* mutants and WT Col-0, 200 fully elongated root hairs were measured ($n = 30$ roots) from seedlings grown on vertical plates for 10 d. Values are reported as the mean $\pm$ SD using ImageJ 1.50b software (NIH). Measurements made on images were captured with an Olympus SZX7 zoom microscope equipped with a Q-Color5 digital camera. Ten-day-old roots for ARF^{OE} were digitally photographed with a stereomicroscope (M205 FA; Leica) at a 40 $\times$ magnification. The hair length of 8 to 10 consecutive hairs protruded perpendicularly from each side of the root, for a total of 16 to 20 hairs from both sides of the root.

$\text{H}_2\text{DCF-DA}$ Probe Used to Measure Total ROS. Growth of *Arabidopsis* seeds on a plate was done with 1% sterile agar for 8 d in a chamber at 25 $^\circ\text{C}$ with continuous light. These seedlings were incubated in darkness on a slide for 10 min with 50 μM 2',7'-dichlorodihydrofluorescein diacetate at room temperature. Samples were observed with a confocal microscope equipped with a 488-nm argon laser and BA510IF filter sets. A 10 $\times$ objective was used, 0.30 N.A., 4.7 laser intensity, 1.1 offset, 440 photomultiplier (PMT) (for highest ROS levels), 480 PMT (for ROS media), and gain 3. Images were taken scanning XZY with 2 μm between focal planes. Images were analyzed using ImageJ. To measure ROS highest levels, a circular region of interest (ROI) ($r = 2.5$) was chosen in the zone of the root hair with the highest intensities. To

measure ROS mean, the total area of the root hair was taken. Pharmacological treatments were carried out with a combination of the following reagents: 100 nM and 5 μM IAA, 15 μM VAS2870, and 100 μM SHAM. In the case of a short time experiment with IAA, 8-d-old WT plants were incubated with 100 nM IAA for 3 min and coinubated for 7 min with 100 nM IAA and 50 μM $\text{H}_2\text{DCF-DA}$ at room temperature. We washed the sample with MS 0.5 $\times$ solution, and image acquisition was done with a 10 $\times$ objective and 400 ms of exposure time on an epifluorescence microscope (Imager A2; Zeiss). As a control, we incubated 10-d-old WT plants with MS 0.5 $\times$ solution for 3 min and coinubated for 7 min with MS 0.5 $\times$ and 50 μM $\text{H}_2\text{DCF-DA}$ at room temperature. To measure ROS levels, a circular ROI ($r = 2.5$) was taken at the tip of the root hair. Values are reported as the mean $\pm$ SD using ImageJ 1.50b software.

ACKNOWLEDGMENTS. We thank T. Beeckman, A. Costa, L. Cárdenas, I. De Smet, L. Dolan, N. Geldner, U. Grossniklaus, T. Hamann, J.-Y. Kim, J. Kleine-Vehn, and D. Weijers for materials; and the H.-T.C. and J.M.E. laboratories for discussions. We thank A. R. Kornblihtt for advice and an anonymous editor for English copy edition. We thank the Arabidopsis Biological Resource Center (ABRC) (Ohio State University) for providing T-DNA seed lines. J.P.M., A.D.N., and J.M.E. are investigators of the National Research Council (CONICET), Argentina. This work was supported by grants from ANPCyT (PICT2013-003, PICT2014-504, and ICGEB2016; to J.M.E.) and the Mid-Career Researcher Program (2015002633) of the National Research Foundation and the Next-Generation BioGreen 21 Program (Agricultural Genome Center PJ011195) of the Rural Development Administration (to H.-T.C.). H.-S.C. was partially supported by the Stadelmann-Lee Scholarship, Seoul National University.

- Menand B, et al. (2007) An ancient mechanism controls the development of cells with a rooting function in land plants. *Science* 316:1477–1480.
- Datta S, Prescott H, Dolan L (2015) Intensity of a pulse of RSL4 transcription factor synthesis determines *Arabidopsis* root hair cell size. *Nat Plants* 1:15138.
- Slabaugh E, Held M, Brandizzi F (2011) Control of root hair development in *Arabidopsis thaliana* by an endoplasmic reticulum anchored member of the R2R3-MYB transcription factor family. *Plant J* 67:395–405.
- Xu P, et al. (2014) HDG11 upregulates cell-wall-loosening protein genes to promote root elongation in *Arabidopsis*. *J Exp Bot* 65:4285–4295.
- Yi K, Menand B, Bell E, Dolan L (2010) A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. *Nat Genet* 42:264–267.
- Salazar-Henao JE, Schmidt W (2016) An inventory of nutrient-responsive genes in *Arabidopsis* root hairs. *Front Plant Sci* 7:237.
- Song L, et al. (2016) The molecular mechanism of ethylene-mediated root hair development induced by phosphate starvation. *PLoS Genet* 12:e1006194.
- Guilfoyle T, Hagen G, Ulmasov T, Murfett J (1998) How does auxin turn on genes? *Plant Physiol* 118:341–347.
- Fukaki H, Tameda S, Masuda H, Tasaka M (2002) Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of *Arabidopsis*. *Plant J* 29:153–168.
- Bargmann BOR, et al. (2013) A map of cell type-specific auxin responses. *Mol Syst Biol* 9:688.
- Okushima Y, et al. (2005) Functional genomic analysis of the *AUXIN RESPONSE FACTOR* gene family members in *Arabidopsis thaliana*: Unique and overlapping functions of *ARF7* and *ARF19*. *Plant Cell* 17:444–463.
- Foreman J, et al. (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422:442–446.
- Wu J, et al. (2010) Spermidine oxidase-derived H_2O_2 regulates pollen plasma membrane hyperpolarization-activated Ca^{2+} -permeable channels and pollen tube growth. *Plant J* 63:1042–1053.
- Lee Y, Rubio MC, Allassimone J, Geldner N (2013) A mechanism for localized lignin deposition in the endodermis. *Cell* 153:402–412.
- Xie H-T, Wan Z-Y, Li S, Zhang Y (2014) Spatiotemporal production of reactive oxygen species by NADPH oxidase is critical for tapetal programmed cell death and pollen development in *Arabidopsis*. *Plant Cell* 26:2007–2023.
- Orman-Ligeza B, et al. (2016) RBOH-mediated ROS production facilitates lateral root emergence in *Arabidopsis*. *Development* 143:3328–3339.
- Monshausen GB, Bibikova TN, Messerli MA, Shi C, Gilroy S (2007) Oscillations in extracellular pH and reactive oxygen species modulate tip growth of *Arabidopsis* root hairs. *Proc Natl Acad Sci USA* 104:20996–21001.
- Takeda S, et al. (2008) Local positive feedback regulation determines cell shape in root hair cells. *Science* 319:1241–1244.
- Boisson-Dernier A, et al. (2013) ANXUR receptor-like kinases coordinate cell wall integrity with growth at the pollen tube tip via NADPH oxidases. *PLoS Biol* 11:e1001719.
- Passardi F, Penel C, Dunand C (2004) Performing the paradoxical: How plant peroxidases modify the cell wall. *Trends Plant Sci* 9:534–540.
- Brouwer KS, van Valen T, Day DA, Lambers H (1986) Hydroxamate-stimulated O(2) uptake in roots of *Pisum sativum* and *Zea mays*, mediated by a peroxidase: Its consequences for respiration measurements. *Plant Physiol* 82:236–240.
- Schlereth A, et al. (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. *Nature* 464:913–916.
- Galli M, et al. (2015) Auxin signaling modules regulate maize inflorescence architecture. *Proc Natl Acad Sci USA* 112:13372–13377.
- Lee M-S, An J-H, Cho H-T (2016) Biological and molecular functions of two ear motifs of *Arabidopsis* IAA7. *J Plant Biol* 59:24–32.
- Tsukagoshi H, Busch W, Benfey PN (2010) Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. *Cell* 143:606–616.
- Lu D, Wang T, Persson S, Mueller-Roeber B, Schippers JHM (2014) Transcriptional control of ROS homeostasis by KUODA1 regulates cell expansion during leaf development. *Nat Commun* 5:3767.
- Vijayakumar P, Datta S, Dolan L (2016) ROOT HAIR DEFECTIVE SIX-LIKE4 (RSL4) promotes root hair elongation by transcriptionally regulating the expression of genes required for cell growth. *New Phytol* 212:944–953.
- Hwang Y, Choi HS, Cho HM, Cho HT (2017) Tracheophytes contain conserved orthologs of a basic helix-loop-helix transcription factor that modulate ROOT HAIR SPECIFIC genes. *Plant Cell* 29:39–53.
- Sundaravelpandian K, Chandrika NN, Schmidt W (2013) PFT1, a transcriptional mediator complex subunit, controls root hair differentiation through reactive oxygen species (ROS) distribution in *Arabidopsis*. *New Phytol* 197:151–161.
- Yun BW, et al. (2011) S-nitrosylation of NADPH oxidase regulates cell death in plant immunity. *Nature* 478:264–268.
- Nestler J, et al. (2014) Roothairless5, which functions in maize (*Zea mays* L.) root hair initiation and elongation encodes a monocot-specific NADPH oxidase. *Plant J* 79:729–740.
- Kwon T, et al. (2015) Transcriptional response of *Arabidopsis* seedlings during space-flight reveals peroxidase and cell wall remodeling genes associated with root hair development. *Am J Bot* 102:21–35.
- Dunand C, Crèvecoeur M, Penel C (2007) Distribution of superoxide and hydrogen peroxide in *Arabidopsis* root and their influence on root development: Possible interaction with peroxidases. *New Phytol* 174:332–341.
- Dynowski M, Schaaf G, Loque D, Moran O, Ludewig U (2008) Plant plasma membrane water channels conduct the signalling molecule H_2O_2 . *Biochem J* 414:53–61.
- Miller EW, Dickinson BC, Chang CJ (2010) Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proc Natl Acad Sci USA* 107:15681–15686.
- Tian S, et al. (2016) Plant aquaporin AtPIP1;4 links apoplastic H_2O_2 induction to disease immunity pathways. *Plant Physiol* 171:1635–1650.