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SHORT COMMUNICATION

The lncRNA *APOLO* and the transcription factor WRKY42 target common cell wall EXTENSIN encoding genes to trigger root hair cell elongation

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ABSTRACT

Plant long noncoding RNAs (lncRNAs) are key chromatin dynamics regulators, directing the transcriptional programs driving a wide variety of developmental outputs. Recently, we uncovered how the lncRNA *AUXIN REGULATED PROMOTER LOOP* (*APOLO*) directly recognizes the locus encoding the root hair (RH) master regulator *ROOT HAIR DEFECTIVE 6* (*RHD6*) modulating its transcriptional activation and leading to low temperature-induced RH elongation. We further demonstrated that *APOLO* interacts with the transcription factor WRKY42 in a novel ribonucleoprotein complex shaping *RHD6* epigenetic environment and integrating signals governing RH growth and development. In this work, we expand this model showing that *APOLO* is able to bind and positively control the expression of several cell wall EXTENSIN (EXT) encoding genes, including *EXT3*, a key regulator for RH growth. Interestingly, *EXT3* emerged as a novel common target of *APOLO* and WRKY42. Furthermore, we showed that the ROS homeostasis-related gene *NADPH OXIDASE C (NOXC)* is deregulated upon *APOLO* overexpression, likely through the RHD6-RSL4 pathway, and that *NOXC* is required for low temperature-dependent enhancement of RH growth. Collectively, our results uncover an intricate regulatory network involving the *APOLO*/WRKY42 hub in the control of master and effector genes during RH development.

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Root hairs; long noncoding RNAS; *APOLO*; EXTENSINs; NADPH oxidase C (NOXC); RHD6; WRKY42; low temperature

Over the last few years, long noncoding RNAs (lncRNAs) have emerged as key regulators of a diversity of biological and molecular processes. In particular, the lncRNA *APOLO* (*AUXIN REGULATED PROMOTER LOOP*) has been recently implicated in 3D chromatin conformation dynamics of multiple spatially unrelated loci across the *Arabidopsis* genome.[1,2] It has been proposed that *APOLO* regulates the expression of a myriad of auxin-responsive genes in roots via sequence complementarity and R-loop formation, not only in *cis* (i.e. its neighboring gene *PINOID*) but also in *trans*.[1–3] More recently, we have uncovered a new molecular mechanism involving *APOLO* action in root hair (RH) polar growth under low temperature (10°C).[4] Shortly, we described how *APOLO* can regulate the shape of a chromatin loop encompassing the promoter region of *Root Hair Defective 6* (*RHD6*), a master regulator of RH initiation.[5] Thus, *APOLO* regulates directly the transcription of *RHD6* and indirectly the expression of *RHD6* and/or auxin downstream RH-related genes, including key factors like *RHD6-like 4* (*RSL4*) and *RHD6-like 2 (RSL2)*, which ultimately promote an exacerbated RH cell elongation under cold temperatures. Furthermore, we identified the WRKY42 protein (belonging to the WRKY family of transcription factors containing the WRKYGQK motif), previously linked to phosphate homeostasis,[6] as an *APOLO* direct partner in the cell nucleus. Strikingly, we demonstrated that the *APOLO*/WRKY42 ribonucleoprotein complex can shape the epigenetic landscape of the *RHD6* locus to activate a transcriptional reprogramming under cold, in an *APOLO*-dependent stoichiometric mechanism. In addition, we found a polycomb-dependent dynamic deposition of the silencing mark H3K27me3 over the *RHD6* promoter region as an additional layer of gene expression regulation under low temperature.[4] Notably, the inclusion of the inorganic phosphate (Pi) starvation-related factor WRKY42 as part of this epigenetic regulatory mechanism suggested that low temperature might not be the major triggering factor of RH cell elongation phenotype. Thus, we provided evidence indicating that the nutrient mobility restriction linked to low temperatures may constitute the key factor promoting a major increase in RH cell elongation. Nevertheless, further research will be required to elucidate which nutrient(s) mediate(s) this effect of cold on RH growth.

In this study, we identified in addition to *RHD6*, 16 RH-related genes among the 187 *APOLO* direct *bona fide* targets, according to publicly available *APOLO*-ChIRP-Seq datasets.[2] Out of these 16 targets, 11 genes encode cell wall EXTENSINs (EXTs) and EXT-related proteins. In addition, we found 7 EXTs and EXT-related encoding genes (PRPs for Proline Rich Proteins, LRXs for Leucine-Rich Extensin Proteins and PERKs for Proline-rich Extensin-like Receptor Kinases) as

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APOLO indirect targets, that is, not directly bound by *APOLO* but transcriptionally deregulated in the *35S:APOLO* seedlings compared to Col-0 wild-type (Figure 1(a)). Notably, most of *APOLO* direct and indirect RH-related targets appeared as transcriptionally activated in the *35S:APOLO* background, hinting a positive regulation mediated by *APOLO*. Among *APOLO* direct targets, we selected *EXT3*, exhibiting a similar behavior as *RHD6* and bearing four potential WRKY binding sites in its promoter region, to further highlight the *APOLO-dependent* regulation of RH-related genes downstream *RHD6*. It was previously reported that EXT3 is involved in the cell plate (i.e. the nascent cross wall) formation during cytokinesis of the embryo and the *ext3* null mutant is embryo lethal.[9] The epigenetic profile of the *EXT3* locus (AT1G21310) corresponds to a typical *APOLO* target[2] (Figure 1(b)), including LHP1 recognition (track 1, chromatin immunoprecipitation (ChIP)-Seq), H3K27me3 deposition (track 2),[7] and *APOLO* binding regions[2] (tracks 3 to 5, chromatin isolation by RNA purification (ChIRP)-Seq). In addition, a peak of DNA-RNA hybrid immunoprecipitation (DRIP)-Seq from root samples indicates the presence of an R-loop coinciding with *APOLO* recognition sites over *EXT3*[8] (tracks 6 to 9). Altogether, our results indicate that *APOLO* lncRNA directly regulates *EXT3* transcriptional activity by sequence complementarity and R-loop formation. Interestingly, several *APOLO*-targeted *EXT* genes contain W-boxes (*TTGACY* sequence) the first 2500 bp upstream of the ATG, an indicative of being putative targets of WRKY42 (Figure 1(a)) as demonstrated for *RHD6*.[4] Therefore, we confirmed by ChIP the binding of WRKY42 to the *EXT3* promoter in Arabidopsis plants stably transformed with the *35S:WRKY42:GFP* construct (Figure 1(c)). Furthermore, we assessed the impact of *APOLO* deregulation over WRKY42 recognition of the *EXT3* promoter. To this end, we transiently transformed leaves of RNA*i-APOLO* or *35S:APOLO* plants with the *35S:WRKY42:GFP* to perform a comparative ChIP-qPCR as previously described.[4] Interestingly, *APOLO* silencing or over-accumulation can impair WRKY42 binding to its target promoter, further supporting the stoichiometric role of *APOLO* over its protein partner activity. In agreement with our previous model proposing that *APOLO* dynamically

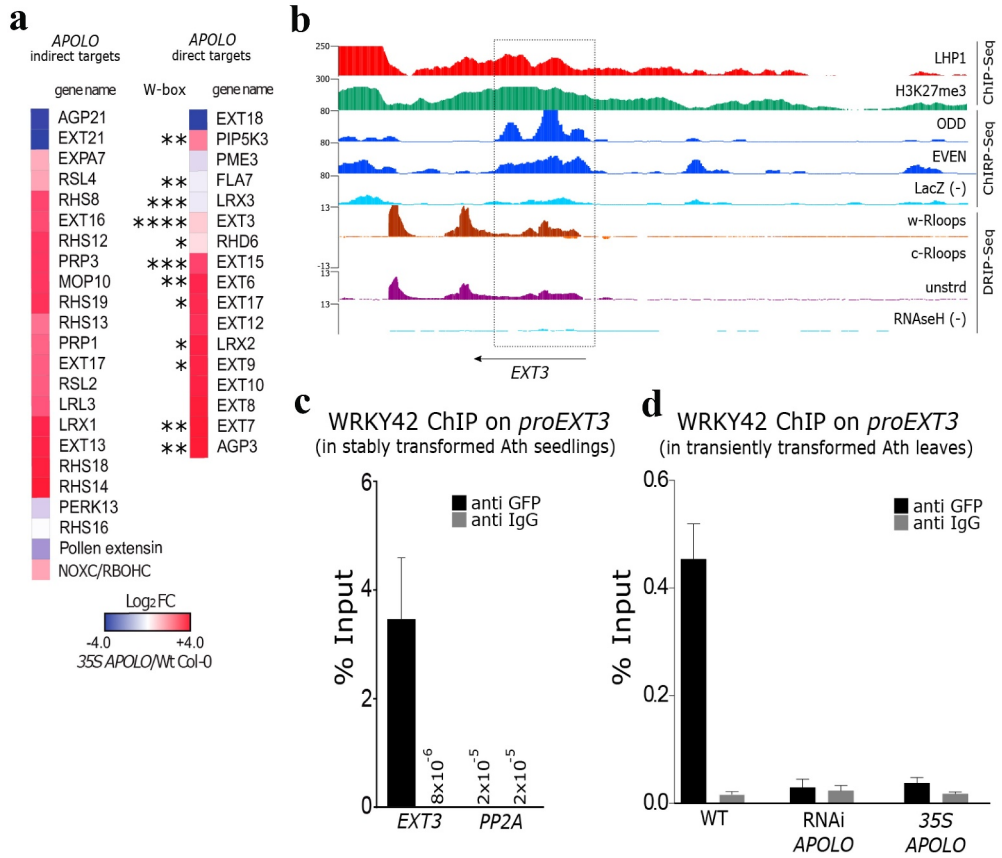

Figure 1. *APOLO* and WYRK42 directly and indirectly control the expression of several cell wall EXTENSIN encoding genes required for RH growth. (a) Heat map of plant cell wall EXTENSINs and related glycoproteins as APOLO targets and non-targets mostly upregulated in 35S-APOLO vs Wt Col-0 as Log_2FC. With asterisks are indicated the number of putative W-boxes in their regulatory regions (2.5 kb). The TFs RHD6, RSL4 and RSL2 are also included together with other specific root hair specific (RHS) genes. NOXC (also known as RHD2/RBOHC) is also upregulated by APOLO (by 2.6 Log_2FC). (b) Epigenomic landscape of the *EXT3* and *NSRb* loci. Track 1: LHP1 deposition by ChIP-Seq.[7] Track 2: H3K27me3 deposition by ChIP-Seq.[7] Tracks 3 to 5: *APOLO* recognition by ChIRP-Seq (Lane 3 and 4, using ODD and EVEN sets of probes against *APOLO*, respectively; Track 5, negative control using LacZ probes).[2] Tracks 6 to 8: R-loop formation by DRIP-Seq (R-loop Atlas, root samples,[8] on Watson strand (Track 6) and Crick strand (Track 7). DRIP negative control after RNAseH treatment is shown in Track 8. Gene annotation is shown at the bottom. (c) Chromatin Immunoprecipitation (ChIP)-qPCR assay in stably transformed *Arabidopsis thaliana* seedlings revealing regulation by WRKY42 of *EXT3* by direct recognition of its promoter region. Probes amplifying *PP2A* were used as a negative control of the experiment. Anti-IgG antibodies were used as a negative control for each pair of probes. (d) Chromatin Immunoprecipitation (ChIP)-qPCR assay in transiently transformed *Arabidopsis thaliana* seedlings (WT), RNAi-*APOLO* (low levels of *APOLO*) and *35S:APOLO* (high levels of *APOLO*) lines revealing an *APOLO*-dependent interaction between WRKY42 and *EXT3* promoter region. Anti-IgG antibodies were used as a negative control for each pair of probes.

recruits WRKY42 to *RHD6*,[4] here we show that the *APOLO*/WRKY42 hub regulates additional RH-related genes. Likely, the expression of a larger subset of EXTs encoding genes including *EXT3* may be coordinately modulated by *APOLO* and WRKY42, together with the previously demonstrated common target *RHD6*.[4]

EXTs are a large group of cell wall *O*-glycosylated proteins belonging to the Hydroxyproline Rich Family (HRGP) superfamily (for details, see[10–14]). In *Arabidopsis thaliana* EXT-related glycoproteins are encoded by up to 59 genes.[12,13,15,16] EXTs usually contain in their sequences multiple Ser-$(Pro)_{3-5}$ repeats that may be *O*-glycosylated and Tyr (Y)-based motifs that could be cross-linked.[17] Monomeric secreted EXTs form rod-like structures with a polyproline-II helical conformation[18–21] that allow them to form an extended EXT-network at the nascent cell walls.[9] On the other hand, secreted Class-III peroxidases (Class-III PRXs) are thought to facilitate EXTs both intramolecular and intermolecular covalent Tyr-Tyr cross-links.[22–24] Recently, we identified three Class-III PRXs (PRX01, PRX44, PRX73) that are highly expressed in growing RHs and might be involved in EXT-crosslinking during cell expansion;[25,26] however, the underlying molecular mechanisms are not completely determined. Interestingly, several of the *APOLO* targeted *EXTs* identified here are crucial for RH cell elongation and also they are regulated by RSL4,[17,20,27–30] which appeared as a key factor in the response to cold.[4] Therefore, *APOLO* and WRKY42 participate in an intricate regulatory network controlling the expression of master as well as effector genes in RH cell elongation.

Remarkably, the *RBOHC* gene (for *Respiratory Burst Oxidase protein C*; also named as *NADPH Oxidase C, NOXC* or *RHD2*, for *Root Hair Defective 2*), a crucial component in the ROS production of growing RHs,[25,31] was also upregulated in *35S:APOLO* seedlings,[4] although it was not identified as a direct target (Figure 1(a)). We have previously proposed that auxin controls RH polar growth through the transcription factor RSL4 activating downstream *RBOHC* transcription and ROS production.[25] *RSL4* expression is regulated by auxin via several AUXIN RESPONSE FACTORS including ARF5, ARF7 and ARF19, which then induces the expression of downstream target genes, such as *NADPH oxidases*, including *RBOHC/NOXC* and *NOXJ*, as well as 4 Class-III *PRXs* (*PRX01, PRX44, PRX60* and *PRX73*). Notably, it has been shown that RSL4 directly controls the expression of *RBOHC/NOXC* and *NOXJ*.[25] Both NOXs and Class-III PRXs regulate the ROS homeostasis in the apoplast of RHs.[17,25] Remarkably, *rbohc-1* mutant seedlings (Salk_071801 T-DNA knockout mutant characterized previously[32–34]) show a reduced RH length in standard conditions as well as under low temperatures, in contrast to wild-type Col-0 plants (Figure 2(a)). These results indicate that RBOHC/NOXC is required to maintain the proper ROS homeostasis during the RH cell elongation under low temperatures. Notably, it has been shown that ROS production is important to modify the dynamic status (assembly/disassembly) of the growing cell wall polymers including the EXT-crosslinking catalyzed by specific Class-III PRX.[17,26]

Altogether, our novel results expand the understanding about the role of the *APOLO*/WRKY42 regulatory hub recently identified[4] in the context of the RH developmental program.[17] According to our observations, *APOLO*, WRKY42 and the *APOLO*/WRKY42 complex are able to control the expression of key players during RH growth under low-temperature conditions (10°C), including the master regulator of RH initiation *RHD6*, which activates the key genes *RSL4/RSL2*, together with a subset of cell wall glycoprotein *EXTs* and ROS-producer *RBOHC/NOXC* (Figure 2(b)). When perturbing *APOLO* and WRKY42, we did not detect changes in RH density, RH initiation process or in RH cell shape, highlighting their major role in RH cell elongation.[4] Further research will be required to determine whether *APOLO* interacts with other TFs to form diverse regulatory complexes in order to integrate environmental and developmental cues into the coordinated regulation of gene expression in Arabidopsis.

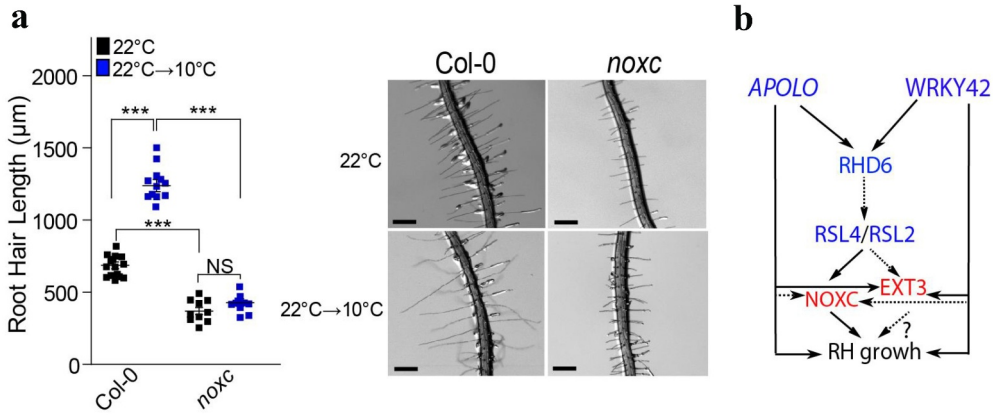

Figure 2. Low-temperature-dependent RH growth requires NOXC to trigger RH cell elongation. (a) Quantification of RH length of plants Col-0 and *noxc* mutant at 22°C (black squares) and 10°C (blue squares) on the left. Each point is the mean ± error of the length of the 10 longest RHs identified in the root elongated zone in a single root. Representative images of each genotype are shown on the right. Scale bars represent 400 µm. Statistical analysis corresponds to a one-way ANOVA followed by a Tukey–Kramer test; *p-value* <0.05. NS stands for non-significant. (b). Simplified working model of *APOLO* + WRKY42 control of RH growth by enhancing the expression of *RHD6* and downstream genes such as *EXT3* (cell wall) and *RBOHC/NOXC* (ROS homeostasis). In blue are indicated the low-temperature inducible components so far characterized. Solid lines indicate direct regulation. Dotted-lines indicates indirect or potentially direct (to be confirmed experimentally) regulation. The question mark "(?)" between EXT3 and RH growth indicates that the corresponding phenotype has not been experimentally validated yet.

Materials and methods

Root hair phenotype characterization

For quantitative analyses of RH phenotypes, 10 fully elongated RH from the root elongated zone of 15–20 roots were measured on the same conditions for each particular case and grown on vertical plates with ½-strength Murashige and Skoog media (1/2 MS) (Duchefa, Netherlands) and 0.8% plant agar (Duchefa, Netherlands) for 5 d at 22° and 3 d at 10°C. Measurements were made after 8 d. The images were captured with an Olympus SZX7 Zoom Stereo Microscope (Olympus, Japan) equipped with a Q-Colors digital camera and software Q Capture Pro 7 (Olympus, Japan). Results were expressed as the mean ± standard error (SE). All measurements indicate the average of three independent experiments.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed on 10-d-old WT seedlings treated or not during 24 h at 10°C, using anti H3K27me3 (Diagenode pAb-195-050), anti LHP1 (Covalab pab0923-P) and anti-IgG (Abcam ab6702) as described before.[2] Crosslinked chromatin was sonicated using a water bath Bioruptor Pico (Diagenode; 30 sec ON/30 sec OFF pulses; 10 cycles; high intensity). ChIP was performed using Invitrogen Protein A Dynabeads. Precipitated DNA was recovered using phenol:chloroform:isoamilic acid (25:24:1; Sigma) and subjected to RT-qPCR. Untreated sonicated chromatin was processed in parallel and considered the input sample. After regular chromatin isolation from 10-d-old *35S:WRKY42:GFP* seedlings, the sample was split in four independent tubes and diluted to 1 ml in Nuclei Lysis Buffer without SDS. Then, cross-linking was performed with 1% formaldehyde for 5 min at 4°C, followed by 5 min with a final concentration of 50 mM glycine. SDS was added to a final concentration of 0.1% prior to sonication and the subsequent steps of a regular ChIP protocol. For ChIP in transiently transformed leaves, 3-week-old *A. thaliana* were transformed as previously described.[35] In brief, *Agrobacterium tumefaciens* strain GV3101 carrying *35S:WRKY42:GFP* construct was grown for 2 d in YEB-induced medium plates at 28°C. Agrobacterium cells were scraped and resuspended in washing solution (10 mM $MgCl_2$, 100 µM acetosyringone). Infiltration solution (¼MS [pH = 6.0], 1% sucrose, 100 µM acetosyringone, 0.005% [v/v, 50 µl/l] Silwet L-77) was prepared with the previous solution, adjusting the OD600 = 0.5. The infiltration was carried out in all leaves >0.5 cm in length of between 10 and 15 plants per genotype. After infiltration, plants were kept in light for 1 h and then in darkness for 24 h. Finally, they were transferred back to light. Samples were obtained 3 d after infiltration.

A heatmap on *APOLO* direct and indirect targets was made with Morpheus (https://software.broadinstitute.org/morpheus/) based on the data published.[4]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

JMP, MM, LL, NM, VBG performed the experiments. JMP, FA, and JE analyzed the data. FA and JE conceived the project. JMP, FA, and JE wrote the manuscript with the contribution of all authors.

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