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The long intergenic noncoding RNA *ARES* modulates root architecture in Arabidopsis

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Abstract

Long noncoding RNAs (lncRNAs) have emerged as important regulators of gene expression in plants. They have been linked to a wide range of molecular mechanisms, including epigenetics, miRNA activity, RNA processing and translation, and protein localization or stability. In Arabidopsis, characterized lncRNAs have been implicated in several physiological contexts, including plant development and the response to the environment. Here we searched for lncRNA loci located nearby key genes involved in root development and identified the lncRNA *ARES* (*AUXIN REGULATOR ELEMENT DOWNSTREAM SOLITARYROOT*) downstream of the lateral root master gene *IAA14/SOLITARYROOT* (*SLR*). Although *ARES* and *IAA14* are co-regulated during development, the knockdown and knockout of *ARES* did not affect *IAA14* expression. However, in response to exogenous auxin, *ARES* knockdown impairs the induction of its other neighboring gene encoding the transcription factor NF-YB3. Furthermore, knockdown/out of *ARES* results in a root developmental phenotype in control conditions. Accordingly, a transcriptomic analysis revealed that a subset of *ARF7*-dependent genes is deregulated. Altogether, our results hint at the lncRNA *ARES* as a novel regulator of the auxin response governing lateral root development, likely by modulating gene expression in *trans*.

Abbreviations: ARES, Auxin regulator element downstream solitaryroot; ARF, Auxin Response Factor; Aux/IAA, Auxin/Indole-3-Acetic Acid; DCL3, Dicer-like 3; DEG, differentially expressed genes; GO, Gene Ontology; HPA1, Histidinol phosphate aminotransferase 1; HSP20-like, Heat shock protein 20-like; IAA1, Indole-3-acetic acid inducible 1; IAA3, Indole-3-acetic acid inducible 3; IAA14, Indole-3-acetic acid inducible 14; IAA19, Indole-3-acetic acid inducible 19; lincRNA, long intergenic noncoding RNA; lncRNA, long noncoding RNA; NF-YB3, Nuclear factor Y, Subunit B3; NAA, 1-Naphthaleneacetic acid; NAT, natural antisense transcript; NPA, Naphthylphthalamic acid; NRPD2A, Nuclear RNA polymerase D2A; NRPD1A, Nuclear RNA polymerase D1A; PIP5K, Phosphatidylinositol 4-phosphate 5-kinase; RNAi, RNA interference; RdDM, RNA-directed DNA methylation; SHY2, Short hypocotyl 2; SLR, Solitaryroot; TSS, Transcription Start Site.

KEYWORDS

ARES, ARF7, AUXIN, lateral root development, long noncoding RNA, SOLITARYROOT

1 | INTRODUCTION

During the last few years, plant long noncoding RNAs (lncRNAs) have been linked to a wide variety of molecular mechanisms modulating gene expression, ranging from epigenetics and transcription, to translation and protein modification.[1] Nuclear-enriched lncRNAs regulate gene expression through diverse mechanisms, including epigenetics, splicing, and subcellular localization of protein partners.[1,2] Some lncRNAs act in *cis*, by recruiting histone-modifying complexes,[3,4] by RNA Polymerase II (Pol II) collision of convergent genes,[5] by influencing the stability or translation of its overlapping gene[6] or by modulating chromatin 3D conformation.[7,8] On the other hand, *trans*-acting lncRNAs may act through DNA–RNA or protein-RNA duplex formation for the regulation of gene expression.

The lncRNA *APOLO* is known to regulate its neighbor gene *PID* by dynamically modulating chromatin 3D conformation in response to auxin.[7] Furthermore, *APOLO* is transcribed by Pol IV and V, and subjected to RNA-directed DNA methylation (RdDM), supporting that *APOLO* methylation status can affect its function on chromatin dynamics. More recently, it was shown that *APOLO* can recognize multiple independent loci across the Arabidopsis genome by sequence complementarity and R-loop formation. *APOLO* can modulate target chromatin conformation dynamics by interaction with Polycomb proteins[9] and transcription factors.[10] As a result, *APOLO* regulates auxin-responsive lateral root development and the expansion of root hair cells at low temperatures.

Here we browsed through the *Arabidopsis thaliana* genome in search of *APOLO*-like lncRNAs to assess if they perform *cis* and/or *trans* actions. To this end, we looked for loci transcribed into lncRNAs in Arabidopsis roots, as previously identified.[11] We focused on precursors of 24 nt siRNAs, that is, controlled by RdDM. Finally, we selected those whose neighbors are known players in root development. After analyzing the co-transcriptional accumulation of adjacent genes along Arabidopsis development, we chose a novel lncRNA for further characterization. The *AUXIN REGULATOR ELEMENT DOWNSTREAM SOLITARYROOT* (*ARES*) is encoded downstream of the lateral root master regulator *IAA14/SLR*,[12] and both loci are regulated during LR development. However, *ARES* knockdown does not affect *IAA14* gene expression although these plants exhibit a lateral root developmental disorder, in agreement with the transcriptomic analysis uncovering deregulated genes. Altogether, our study hints at *ARES* as a *trans*-acting lncRNA participating in lateral root development.

2 | RESULTS

2.1 | The locus *ARES* is regulated by RdDM and is transcribed into a nuclear-enriched long noncoding RNA co-regulated with its neighbor gene *SOLITARYROOT*

Among the 1,671 intergenic lncRNAs (or lincRNAs) detected in *Arabidopsis thaliana* root tips,[11] 456 co-localize with 24 nt siRNAs (Table S1[11]), potentially regulated through RdDM and affecting the activity of neighboring genes. Interestingly, five lincRNAs/siRNA precursors are neighbors to root-related coding genes (Tables S1 and S2, shortlist derived from the literature[13]): *XLOC_005697*, *XLOC_002421*, *AT1G48625*, *AT2G34655* (*APOLO* lncRNA), and *AT4G14548/ARES* (Figure 1a). Using the Genevestigator database,[14] we explored the expression of the shortlisted lincRNAs and their respective neighbor coding genes in roots, and their potential co-regulation during plant development. *XLOC_005697* and *XLOC_002421* are not included in the official Arabidopsis annotations and thus were not available in this database. The lncRNA encoded by *AT1G48625* was only detected in inflorescences, thus poorly correlated with its neighbor gene *AT1G48630*/*RACK1B* which is expressed ubiquitously during plant development (Figure S1). Interestingly, the lincRNA *ARES* and its closest neighbor gene *AT5G14550* (*IAA14/SLR*) are detected in roots and expressed throughout the life of the plant and to a lesser extent in reproductive-related organs, including flower primordium, flower, siliques, and seeds (Figure 1b,c). Furthermore, analysis of publicly available transcriptomic datasets (TraVa) revealed that the *ARES* lincRNA transcriptional accumulation significantly and positively correlates with that of its coding neighboring genes, that is, *NF-YB3* and *IAA14* (Figure 1d; *AT4G06195* is not included in the TraVa database). Considering this lncRNA is positioned very close to *IAA14*, we called it *ARES*.

To further confirm that the lincRNA *ARES* is regulated through RdDM, we measured its expression in mutant plants altered in this process. As expected, the expression of *ARES* was significantly higher in the *nrpd2a* (common subunit of Pol IV and V), *nrpd1a4* (subunit of Pol IV) and *dcl3* mutant lines, providing additional evidence that this locus is controlled by RdDM

(Figure S2). The poor coding capacity of the lincRNA *ARES* was confirmed by independent coding potential estimators, and compared to the already described *MARS* lncRNA[4] (Figure 1e). In addition, we found that this transcript is accumulated in the cell nucleus (Figure 1f), hinting at a possible role in epigenetic regulations, chromatin action or alternative splicing.

2.2 | The lncRNA *ARES* regulates root growth

To determine whether the transcriptional activity of the *ARES* lncRNA may affect its neighbor genes in *cis*, we isolated homozygous lines of one SALK insertional mutant [*SALK_113294*; located prior ARES TSS[15,16]

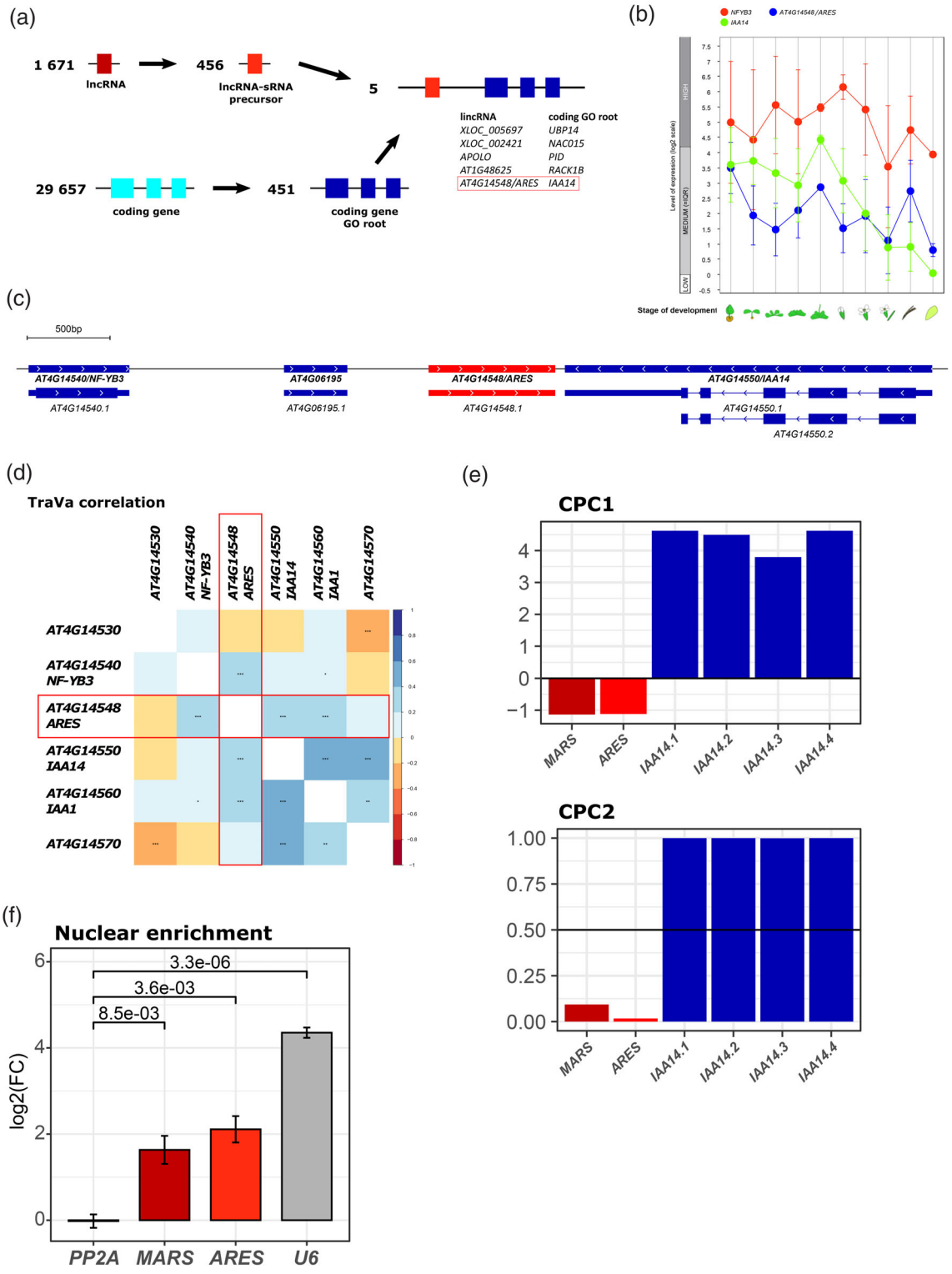

FIGURE 1 Legend on next page.

(Figure S3A)] and three independent knockdown lines by RNAi, targeting *ARES* 5′ region (Figure S3A). *ARES* transcript abundance was reduced in all the lines as compared to Col0. Interestingly, Pol II binding was significantly reduced in the RNAi-*ARES* 3.2 as compared to Col0, suggesting that the RNAi constructs may have reduced the transcription of *ARES* lncRNA in addition to its post-transcriptional degradation (Figure S3B). Despite changes in *ARES* transcriptional activity upon silencing, none of these lines showed a deregulated expression of the two adjacent coding genes, *IAA14* nor of *NF-YB3*, in control conditions (Figure 2a). The expression of the neighboring lncRNA *AT4G06195* remains also unaffected upon *ARES* misregulation (Figure S3C). Considering the role of *IAA14* in auxin-dependent lateral root development, we also assessed the transcriptional response of *ARES* and its neighbor genes during a lateral root induction kinetics (NPA/NAA treatment[17]). Interestingly, both *IAA14* and *ARES* transcript abundance increased, reaching a maximum at 6 h of treatment, while *NF-YB3* transcript abundance decreases with the treatment (Figure 2b). Upon *ARES* knockdown, *IAA14* conserved the same expression level and pattern along the lateral root induction kinetics likewise for *IAA1*, a homolog gene encoded upstream of *IAA14*. Strikingly, *NF-YB3* was significantly up-regulated by the NPA treatment compared to WT but continued with the same level of expression and pattern as in WT in the other time points of the lateral root induction kinetics. Additionally, when plants were treated with NAA alone (without prior application of NPA), the transcript levels of *ARES*, *IAA14*, and *IAA1* were found to increase, whereas the transcript abundance of *NF-YB3* remained unchanged, in three out of four lines (Figure S4). The up-regulation observed only in the ARES-RNAi 6.2 lines may be due to the specific RNAi transgene insertion of this line. In agreement, RNAi-*ARES* lines and the insertional mutant displayed similar responses to the treatment. Altogether, our results indicate that this noncoding transcript does not regulate *IAA14* in *cis*, but may have a direct or indirect effect on *NF-YB3* activity under NPA treatment.

Although *ARES* knockdown did not affect *IAA14* expression in our condition, their co-regulation in plant organs and NPA-NAA treatment (Figures 1d and 2b) made us wonder if this lncRNA may participate in lateral root development. Thus, we characterized root architecture in control conditions. Interestingly, while primary root length was unaffected, average lateral root length was slightly reduced in RNAi lines 1.2 and 3.2 and the insertional mutant, and significantly reduced in RNAi 6.2, the line showing one of the lowest levels of *ARES* (Figure 2c). In agreement, lateral root density was significantly affected in the insertional mutant with low levels of *ARES* (Figure 2c). Altogether, our results suggest that the auxin-responsive lncRNA *ARES* participates in lateral root development. Notably, *ARES*-related phenotypes were observed in control conditions, in which the transcriptional activity of its neighbor genes *IAA14* and *NF-YB3* remain unaffected (at least in whole roots, as harvested here), suggesting that *ARES* regulatory role may not be mediated by a mechanism occurring in *cis*.

2.3 | The root transcriptome of *ARES* knockdown plants uncovers a link to auxin response and lateral root development

To better understand the role of *ARES* in root development, we investigated the full transcriptome of roots of RNAi line 6.2 through RNA-Seq. 1,665 genes were differentially accumulated upon *ARES* knockdown compared to WT. Gene Ontology (GO) analyses hinted at genes encoding nuclear-enriched proteins (cellular components);

FIGURE 1 The *AUXIN REGULATOR ELEMENT DOWNSTREAM SOLITARYROOT (ARES)* locus is transcribed into a long intergenic noncoding RNA downstream of the *IAA14* gene. (a) Flowchart of identification of lncRNA-siRNA which are adjacent to root-related genes. LncRNAs co-localizing with 24 nt siRNA and the neighbor coding gene with a Gene Ontology (GO) related to root growth and development were collected using the Blein et al (2020)[11] transcriptomic analyses and the GO consortium,[13] respectively. The lncRNAs-siRNA potential precursor neighbors to a root-related gene were shortlisted. The resulting five lncRNAs and the name of the neighboring gene are indicated on the right. (b) Transcript abundance of *ARES* and neighbors' genes throughout plant life. Genevestigator snapshot from the Development condition search tool.[14] (c) IGV snapshot of the *ARES* locus and surrounding neighboring genes. For each gene, exons are indicated with rectangles and introns with solid lines. (d) Pearson correlation analysis derived from transcriptomics data from TraVa datasets. Correlations between two genes are indicated with scores ranging from −1 to +1 where −1 corresponds to a negative correlation and + 1 a positive correlation. A color scale indicates the Pearson correlation score. Each correlation was tested for significant differences (* for $p \leq 0.05$, ** for $p \leq 0.01$, *** for $p \leq 0.001$). (e) Coding potential of the *ARES* transcript. Scores were determined using CPC1 and CPC2 (left to right) algorithms.[45,46] For each analysis, the threshold between coding and noncoding genes is displayed with a horizontal dashed black line. Coding genes are situated above the threshold, whereas noncoding genes are situated under. *MARS* is used as positive control for noncoding transcripts. (f) Nuclear enrichment of *ARES*. The housekeeping transcript *PP2A* was considered as a negative control and U6 and the lncRNA *MARS* as positive controls of nuclear-enriched transcripts, for comparison.

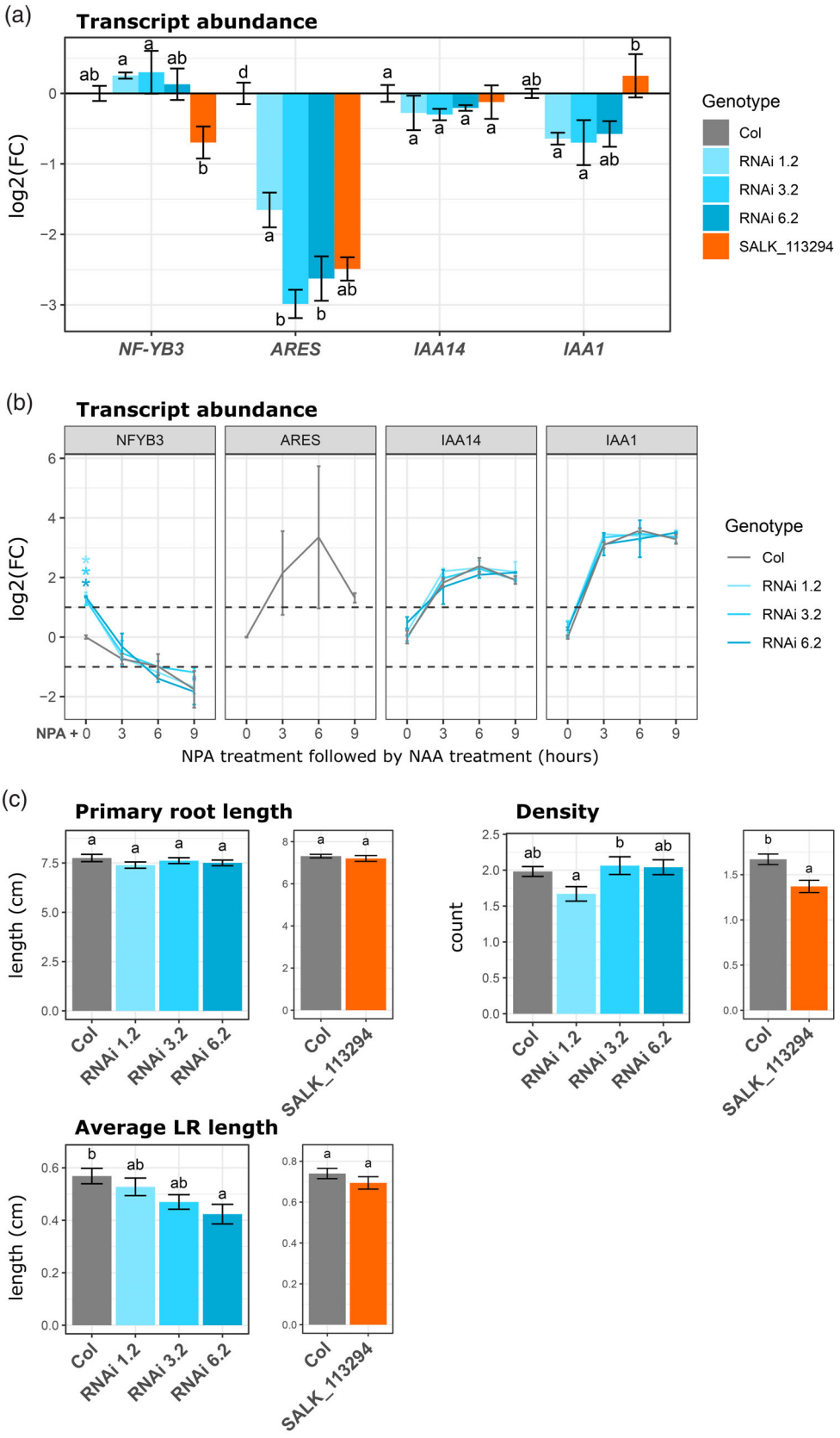

FIGURE 2 The knockdown and knockout of the lncRNA *AUXIN REGULATOR ELEMENT DOWNSTREAM SOLITARYROOT (ARES)* result in an altered root architecture without affecting the expression of *IAA14*. (a) Transcript abundance of *ARES* and its surrounding genes in control conditions in RNAi lines targeting *ARES* and the insertional mutant *SALK_113294*. Transcriptional abundance is shown as the mean ± standard error ($n = 3$) of the log2 fold change compared to the Col0 genotype. Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. For each gene, different letters indicate statistical differences between genotypes ($p \leq 0.05$). (b) Transcript levels of *ARES* and its surrounding genes in response to NPA/NAA treatment in RNAi lines targeting *ARES*. Gene expression data are shown as the mean ± standard error ($n = 3$) of the log2 fold change compared to time 0 h. Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical differences between RNAi-ARES and Col-0 ($p \leq 0.05$) are indicated by stars (*) for each gene and time-point. (c) Mean primary root length, lateral root length, and lateral root density according to the genotype of 12-day-old seedlings. Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's posthoc test. For each genotype, different letters indicate statistical differences ($p \leq 0.05$) between root architecture parameters.

protein and DNA interacting proteins (molecular functions); and notably root development, among several responses to the environment (biological processes; Figure 3a). Interestingly, isoform and splicing variation analyses revealed a change in isoforms and alternative 3′ ends for known root-related genes upon *ARES*

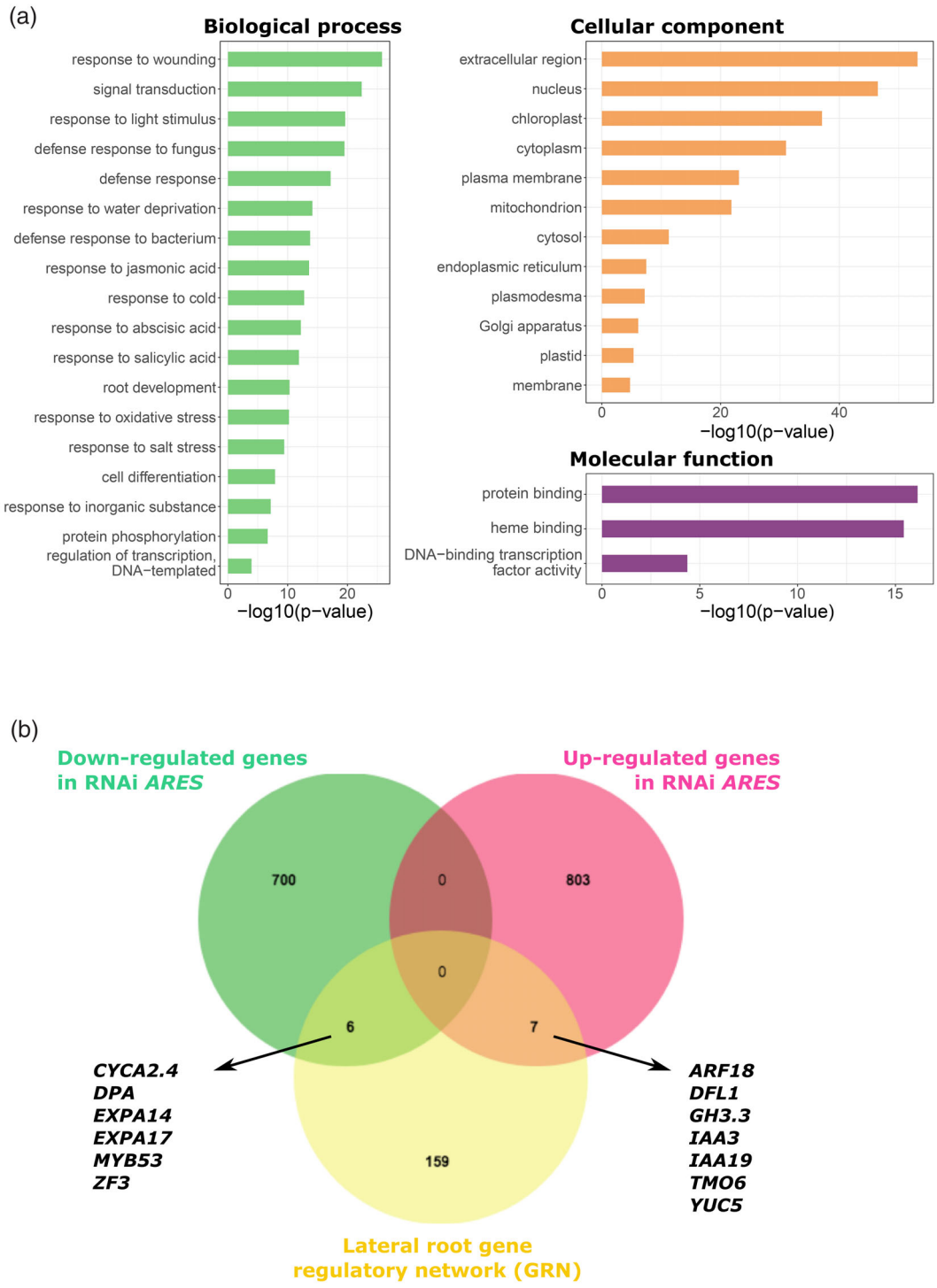

FIGURE 3 The lncRNA *AUXIN REGULATOR ELEMENT DOWNSTREAM SOLITARYROOT (ARES)* regulates auxin-responsive genes involved in lateral root development. (a) Significant enrichment of GO terms in RNAi-*ARES* DEGs for biological processes, cellular components, and molecular function. (b) Overlap between RNAi-*ARES* DEGs (up and down) and genes involved in the lateral root gene regulatory network.[18]

downregulation, including *PIP5K*, *HPA1*, and *HSP20-like*, respectively (Table S3). To further explore the role of *ARES* during lateral root development, we investigated the lateral root gene regulatory network (GRN) defined by Lavenus et al.[18] Among the 172 genes included in these GRN, 13 were deregulated in the RNAi-*ARES* 6.2 line: 6 were downregulated in RNAi-*ARES*, and 7 upregulated (Figure 3b), notably including well-known factors

participating in lateral root development, like *YUCCA5*,[19] *IAA19*[20] and *SHY2/IAA3*,[21] among others. Changes in transcript abundance were confirmed for seven out of eight of these genes in the three RNAi lines, supporting that *ARES* may play a regulatory role in the expression of these root-related genes (Figure S5). Furthermore, when we compared the list of differentially expressed genes (DEG) with available datasets of auxin responses in

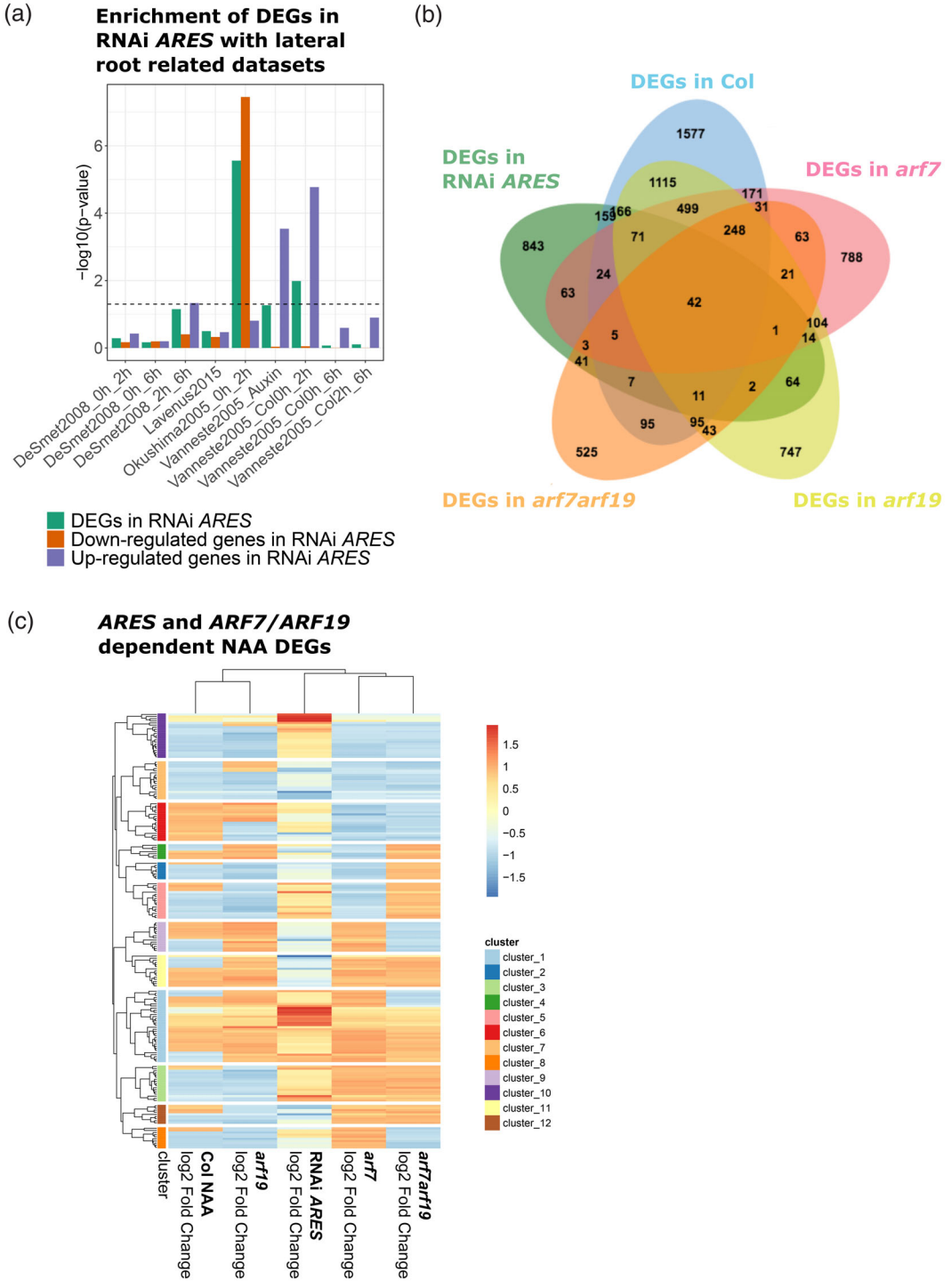

FIGURE 4 AUXIN REGULATOR ELEMENT DOWNSTREAM SOLITARYROOT (*ARES*)-regulated genes are mainly dependent on *ARF7* control in response to auxin. (a) Enrichment of RNAi-*ARES* DEGs (all, down and up) in gene list related to lateral root initiation and development.[18] (b) Overlap between RNAi-*ARES* DEGs with auxin-responsive genes in Col0, *arf7*, *arf19*, *arf7 arf19*. (c) Hierarchical clustering of all RNAi-*ARES* DEGs that are *ARF7* or *ARF19* dependent responsive to auxin based on the scaled log2 fold change in RNAi-*ARES* and NAA treated Col0, *arf7*, *arf19*, *arf7 arf19*. Colors correspond to the scaled log2 fold change in each condition.

Arabidopsis roots, we found a strong enrichment of DEG after a 2 h NAA treatment, derived from two independent works (Figure 4a,[22,23]). In the latest, DEG in response to auxin were particularly overlapped with upregulated genes in RNAi-*ARES* roots. A deeper analysis of the dataset published by Okushima and co-workers[24] allowed us to determine that 45.2% ($n = 488$) of *ARES*-deregulated genes are modulated in response to auxin in Col0, *arf7*, *arf9*, or *arf7-arf19* mutant, whereas 134 genes are dependent on *ARF7/ARF19* (Figure 4b).

Clustering analyses limited to the RNAi-*ARES* DEG, and *ARF7* and/or *ARF19*-dependent auxin-responsive genes ([24]; see Table S4A for genes ID of each cluster), showed that RNAi-*ARES* transcriptome (in control conditions) groups together with *arf7* and *arf7 arf19* double mutant in response to auxin, whereas the profile of genes in *arf19* and WT form a separate group (Figure 4c and Figure S6). Our observations indicate that *ARES* participates in lateral root development in the regulatory pathways dependent on *ARF7*. Furthermore, the clustering analysis revealed that depending on the group of DEG, the link with the response to auxin differs. Cluster 3 groups genes, which behave similarly in RNAi-*ARES* in control conditions and in *arf7* and *arf7/19* roots in response to auxin, whereas these genes exhibit an opposite behavior in WT and *arf19* single mutants under auxin treatment. Cluster 5 group genes are induced in RNAi-*ARES* and *arf7/19* in response to auxin, in contrast to their activity in WT and both single *ARF* mutants. Clusters 10 and 11 group genes, which are induced or repressed upon *ARES* knockdown, respectively, in contrast to an *ARF*-independent response to auxin. Finally, cluster 12 is the only one grouping genes showing a closer behavior between WT, *arf19*, and RNAi-*ARES*, in contrast to *arf7* and *arf7/19*. Altogether, our observations suggest that *ARES* participates in auxin signaling during lateral root development. Altogether, our observations suggest that *ARES* participates in auxin signaling during lateral root development. Additionally, *ARES* downregulation also leads to changes in the splicing of certain genes important for root growth, and the expression of a significant number of auxin-responsive genes, some of which are dependent on *ARF7* and/or *ARF19*.

3 | DISCUSSION

Lateral root development relies largely on auxin homeostasis and the consequent signaling pathways.[25,26] Among the key regulatory genes mediating the action of auxin, we find the TF family of Auxin Response Factors (ARFs) and their related Aux/IAA repressing partners. Twenty-nine Aux/IAA genes are annotated in Arabidopsis and encode short-nuclear proteins involved in the inactivation of the ARF proteins, consequently repressing the auxin transcriptional responses. Auxin stimulus increases the interaction between Aux/IAA proteins and the Skp1-Cullin-F-box/Transport Inhibitor Response 1 (SCF-TIR1) complex, promoting the proteasome-mediated degradation of the Aux/IAA proteins. Notably, gain-of-function mutations of 10 IAA proteins affect plant development. Among them, *iaa14/slr* gain-of-function mutation stops the auxin-mediated cell division in the pericycle, blocking the development of lateral roots.[22,23]

Here, we investigated the noncoding transcriptome of Arabidopsis roots[11] to search for *APOLO*-like RdDM-regulated lincRNAs, which are located next to critical root growth modulator genes, in order to assess if they participate in similar molecular mechanisms as the lncRNA *APOLO*.[7] As a result, we focused on an uncharacterized lncRNA that we named *ARES* located downstream of the lateral root master regulator *IAA14*. Our data shows that *ARES* is regulated during lateral root formation and participates in the regulation of its development through the regulation of lateral root-related genes. Even though our data suggest that *ARES* acts in *trans* we cannot exclude that its function is entirely independent from its neighboring genes *NF-YB3* and/or *IAA14*, or other non-neighboring genes not known to regulate root growth.

Local activity of lncRNAs has been shown for *APOLO*,[7] as well as for other lncRNAs. Recently, the lncRNA *MARS* was implicated in the modulation of the local chromatin conformation in response to ABA, fine-tuning the transcriptional activity of the marneral cluster of genes in Arabidopsis. Likewise *APOLO*, *MARS* directly interacts and titrates the Polycomb protein LHP1 across the region, fine-tuning the epigenetic pattern.[4] However, it remains uncertain if *MARS* can also regulate other genes in *trans*. Several lncRNAs were shown to directly recruit Polycomb proteins, notably the histone methyltransferase CURLY LEAF (CLF), to their transcriptional site, affecting the expression of nearby genes.[4,8,27,28] On the other hand, alternative lncRNAs were also shown to recruit different chromatin-related proteins triggering the deposition of active histone marks H3K4me3 and H4K16Ac in Arabidopsis or rice.[29,30] Although *ARES* deregulation did not strongly affect the transcriptional activity of its adjacent gene *IAA14*, it upregulated the expression of *NF-YB3* under NPA treatment. It is unclear whether *NF-YB3*, which has been shown to enhance heat stress tolerance in plants,[31] also plays a role in regulating lateral root growth, thus we cannot exclude that the *ARES*-mediated *NF-YB3* upregulation does not participate in the auxin-dependent lateral

root growth. Similarly, other genes not known to regulate root growth are deregulated upon *ARES* knockdown and may also participate in the root-related phenotype observed. Furthermore, it is worth noting that similarly to *MARS* and *APOLO*, *ARES* is co-regulated with its closest neighboring gene in response to stress, hinting at an interplay between coding and noncoding transcription and their nearby regulatory elements under stress conditions. In Arabidopsis roots, co- and anti-co-regulation of transcription between coding and noncoding neighboring loci were systematically observed for a subset of lncRNAs in response to phosphate starvation.[11] The regulation of neighbor genes by noncoding transcription was also described for the lncRNA *SVALKA*.[5] Pol II read-through transcription of *SVALKA* results in a cryptic lncRNA overlapping the locus encoding the TF CBF1 on the antisense strand, termed *as CBF1*. *CBF1* transcription is suppressed by Pol II collision with the *SVALKA-asCBF1* lncRNA.

Although convergent transcription may have led to Pol II collision between *ARES* and *IAA14*, no overlap has been observed between the two loci (Figure S3), and notably the knockdown of *ARES* does not affect *IAA14* transcriptional activity. Even though CAGE-seq data indicated an absence of overlap between *ARES* and *IAA14* (Figure S3), further experiments should be performed to confirm it, notably in response to auxin. It is of importance as auxin directly affects polyadenylation site usage of *IAA14* and is crucial for downstream regulation of *ARF7* and *ARF19*.[32] Furthermore, as *IAA14* and *ARES* do not seem to overlap, their RNAs should not form natural antisense transcript (NAT) pairs, which may have eventually led to the translational promotion of *IAA14* like shown for *PHO1;2* and its cognate NAT in rice. The NAT expression promotes the shuttle of the sense–antisense RNA pair towards polysomes.[6]

Other nuclear-enriched lncRNAs have been identified as *trans*-regulatory transcripts. The lncRNA *ASCO* modulates alternative splicing by interacting with several splicing factors, including NSRa/b, PRP8a, and SmD1b. However, the physical interaction between *ASCO* and genes subjected to alternative splicing has not been explored. Notably, the deregulation of *ASCO* and its partners leads to root developmental phenotypes.[26] The lncRNA *HID1* also regulates its target gene *PIF3* in *trans*. *HID1* participates in large ribonucleoprotein complexes whose composition remains unknown, although it has been shown that *HID1* triggers transcriptional repression of *PIF3*.[33] In Arabidopsis, the TE-derived *lincRNA11195* mediates sensitivity to ABA as demonstrated by longer roots and higher shoot biomass in mutant plants when compared to wild-type. However, the molecular basis behind the action of *lincRNA11195* remains unknown.[34]

Here we identified *ARES* for its similarity with *APOLO* profile, that is, (i) an intergenic lncRNA, (ii) precursor of 24 nt siRNAs, and (iii) neighbor of a root-related gene. Interestingly, in contrast to *APOLO*, *ARES* does not seem to regulate its adjacent locus, even though we cannot entirely exclude this possibility notably at the post-transcriptional or translational level. One major difference between *APOLO* and *ARES* is that the former and its neighbor gene *PID* are divergent and they likely share a single promoter encompassed in the intergenic region. In contrast, *ARES* is located downstream *IAA14*, likely not interfering with the *IAA14* promoter. *ARES* vicinity to *IAA14* led us to assess root development in *ARES* knockdown plants (i.e., lines and one insertional mutant). *ARES* knockdown results in lateral root developmental alterations in agreement with the transcriptomic output revealed by RNA-Seq. Our *ARES*-mediated lateral root phenotype, although subtle, is characteristic of many lncRNA modes of action, which adjust gene expression levels rather than completely knocking them out.[1] While only RNAi 6.2 shows a statistically significant decrease in the lateral root length, all lines with reduced levels of *ARES* tend to have shorter lateral roots. Hence, the downregulation of *ARES* fine-tunes key root-related gene expression, resulting in subtle changes in lateral root growth. In particular, DEG in RNAi-*ARES* roots is enriched in auxin-responsive genes, notably including a large subset of genes exhibiting an opposite behavior to their response to exogenous auxin in wild-type plants. In addition, we observed significant changes in the proportions of isoforms of genes that are directly involved in the regulation of root growth, further supporting the role of *ARES* in regulating root growth. Moreover, by comparing RNAi-*ARES* transcriptome to the transcriptional datasets revealing the response to auxin in *arf7 and arf19* mutants, we found that *ARES* may participate in *ARF7*-dependent pathways. Interestingly, IAA14 functions through its interaction with ARF7 and ARF19, notably the stability of the IAA14 protein, rather than its transcript, plays a role in controlling ARF7/19-dependent auxin response. *Solitaryroot* gain of function mutant plants, with increased stability of IAA14, prevent auxin-dependent release of ARF7/19 and blocked LR formation. Thus, one possibility is that the decreased root density in *ARES*-knockdown lines is due to a change in IAA14 protein stability, likely influencing its interaction with ARF7/19 and the resulting root architecture. Therefore, we propose that *ARES* participates in the intricate regulatory network behind the lateral root development event, likely by direct recognition of target loci in *trans*, or alternatively by modulating the action of protein partners in the cell nucleus, such as proteins involved in epigenetic regulation, chromatin accessibility or splicing.

4 | METHODS

4.1 | Screening for *ARES* identification

Coding, lncRNA, and siRNA annotations were collected according to Blein et al (2020)[11] transcriptomics dataset. The root-related GO was collected from the GO consortium[13] and included all the GO terms related to root growth and development (see GO term and identifier in Table S2). *bedtools closest* to default parameter was used to identify the lncRNA-siRNA precursor close to a root-related gene.[35]

4.2 | Plant lines generated and used for this study

All plants used in this study are in Columbia-0 background. RNAi lines of *ARES* were obtained using the pFRN binary vector[36] bearing 250 bp of the 5′ exon of *ARES* gene (see primers in Table S5), initially sub-cloned into the pENTR™/D-TOPO™ vector system from Invitrogen™. Arabidopsis plants were transformed using *Agrobacterium tumefaciens* Agl-0.[37] The T-DNA inserted line *SALK_113294* was provided by the Nottingham Arabidopsis Stock Center (NASC). Homozygous mutants were identified by Polymerase Chain Reaction (PCR) (see primers in Table S5).

4.3 | Growth conditions and phenotypic analyses

For single time-point phenotyping, seeds were sown in plates vertically placed in a growing chamber in long-day conditions (16 h in light 150uE; 8 h in dark; 21°C). Plants were grown on solid half-strength MS medium (MS/2) supplemented with 0.7% sucrose and supplemented with 0.8 g/L agar (Sigma-Aldrich, A1296 #BCBL6182V), buffered at pH 5.6 with 3.4 mM 2-(N-morpholino) ethane sulfonic acid. For root phenotype characterization, the root length was measured at 12 days after sowing (DAS) using RootNav software[38] from images taken with a flat scanner. For the lateral root induction time series, 7 days old seedlings were grown in MS/2 on nylon membranes and transferred to plates containing MS/2 containing 10 μM 1-N-Naphthylphthalamic acid (NPA), an inhibitor of auxin transport and lateral root development,[17] for 3 days and transferred to MS/2 plates containing synthetic auxin (1-Naphthaleneacetic acid NAA) at 10 μM to induce lateral root development. Roots were sampled after 0, 3, 6, and 9 h of NAA treatment. For the NAA treatment, seedlings were sprayed with 10 μM at 12 DAS and collected before and 24 h after the treatment.

4.4 | RT-qPCR

Total RNA was extracted from roots using TRI Reagent™ (Sigma-Aldrich) and treated with DNase (Fermentas) as indicated by the manufacturers. Reverse transcription was performed using 1 μg total RNA and the Maxima Reverse Transcriptase (Thermo Scientific) with oligo-dT primers. qPCR was performed on a Light Cycler 480 with SYBR Green master I (Roche) in standard protocol (40 cycles, 60°C annealing). Primers used in this study are listed in Table S5 and depicted in Figure S3 for *ARES* and *IAA14*. Data were analyzed using the ΔΔCt method using *PROTEIN PHOSPHATASE 2A SUBUNIT A3* (*AT1G13320*) for gene normalization[39] and time 0 for time-course experiments.

4.5 | Nuclear purification

WT seedlings were collected to assess the subcellular localization of RNAs. Chromatin was extracted as previously described.[7] Briefly, 1 g of 12DAS non-crosslinked tissue was smashed to powder in liquid nitrogen and resuspended in 25 mL of EB1 (10 mM Tris pH 8, 0.4 M sucrose, 10 mM $MgCl_2$, 5 mM BME, 0.2 mM PMSF, 1X RNAse inhibitor SIGMA R7397). After 5 min incubation on ice, samples were filtered two times in 70um Falcon cell strainer (Fisher, 352,350). 200 μL of the solution was collected and mixed with 800 μL of TRI Reagent (Sigma-Aldrich) and corresponds to the 'total RNA fraction'. Filtered samples were then centrifuged for 20 min at 2,000 *g* and 4°C. Pellets were resuspended in 10 mL of EB2 (10 mM Tris pH 8, 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM BME, 1% Triton X100, 100 μM PMSF, 1X RNAse inhibitor SIGMA R7397) and centrifuged for 10 min at 2,000 *g* and 4°C. Finally, a sucrose gradient using 600–600 μL of EB3 (10 mM Tris pH 8, 1.7 M sucrose, 2 mM $MgCl_2$, 5 mM BME, 0.15% Triton X100) and EB3 re-suspended samples was performed to purify the chromatin. Purified chromatin was resuspended in 1 mL of TRI Reagent (Sigma-Aldrich) and corresponds to the 'nuclear RNA fraction'. RNA samples were treated with DNase, and reverse transcription was performed using random hexamers prior to qPCR analysis. Data were analyzed using the ΔΔCt method using PROTEIN PHOSPHATASE 2A SUBUNIT A3 (AT1G13320) for gene normalization[39] and the total fraction to assess nuclear enrichment.

4.6 | ChIP pol II

Chromatin immunoprecipitation (ChIP) assays were performed on 12-day-old seedlings using anti-IgG (Cell Signaling 2,729) and Pol II (Abcam ab817) antibodies, as described in Ariel et al (2020).[9] Briefly, 1% formaldehyde crosslinked chromatin was resuspended in a solution containing 10 mM Tris–HCl pH 8, 0.4 M sucrose, 10 mM $MgCl_2$, 5 mM βME, and RNAse. After centrifugation, the cell membrane was lysed in a solution containing 10 mM Tris–HCl pH 8, 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM βME, 1% TRITON X100, and 200 μM PMSF. The chromatin was then placed on a sucrose gradient made with a lower layer of pure buffer (10 mM Tris–HCl pH 8, 1.7 M sucrose, 2 mM $MgCl_2$, 5 mM βME, and 0.15% TRITON X100) and an upper layer of resuspended pellet in the same buffer. The nuclei were resuspended in 300 μL of Nuclei Lysis Buffer (50 mM Tris–HCl pH 8, 0.1% SDS, 10 mM EDTA, and 1 μL of 20 U/μL RNAase-in Promega per sample) and sonicated using a water bath Bioruptor Pico (Diagenode; 30 s on/ 30 s off pulses, at high intensity for 10 cycles). ChIP was performed using Invitrogen Protein A Dynabeads. The immunoprecipitated DNA was recovered using Phenol:Chloroform:Isoamilic Acid (25:24:1; Sigma) and analyzed by qPCR. An untreated sonicated chromatin was processed in parallel and considered the Input sample.

4.7 | Library construction and sequencing

Three biological replicates of 12 DAS whole roots grown in control conditions were collected. RNA samples were extracted using TRI Reagent (Sigma-Aldrich) and treated with DNase (Fermentas) as indicated by the manufacturers. Libraries were processed using Illumina Stranded mRNA Prep library preparation kit following the manufacturer's instructions, starting with one microgram of total RNA. 2 × 75-nt paired-end reads were sequenced on a NextSeq 500 Sequencing System (Illumina). Sequence files generated in this study have been deposited in the NCBI GEO database under the accession GSE207358.

4.8 | Differential expression analysis and clustering

Adapter and poor-quality sequences were trimmed using Trimmomatic and ribosomal sequences were removed using sortMeRNA.[40] Cleaned mRNA reads were aligned on the TAIR10 genome[41] using STAR (version 2.7.2a[42]) with the following arguments: --alignIntronMin 20--alignIntronMax 3,000–outSAMtype BAM SortedByCoordinate. Read overlapping exons were counted per genes using featureCounts from the subread package (v1.6.5,[43]) using strand-specific mode (−s 2 -O -M--fraction). Differential gene expression and GO analysis were performed by edgeR using the DicoExpress package[44] using a linear model with genotype and replicate as explanatory factors. Genes with less than 1 count per million reads (CPM) were filtered out as low counts and raw *p*-values were adjusted with Benjamini & Hochberg method (FDR). Differentially expressed genes were defined as having an adjusted *p*-value lower than 0.05. GO enrichment analysis of DEG was conducted with DicoExpress Enrichment module.

To be able to compare the expression modification induced by *ARES* downregulation (RNA-seq) with the influence of auxin on Col0, *arf7*, *arf19*, and *arf7 arf19*, we used log2 fold change value that we scale per gene. The clustering of the genes and the samples was done using the Euclidean distance between genes and using hierarchical clustering. The number of clusters was determined manually.

4.9 | Bioinformatic analyses of transcriptomic datasets

Coding potential was calculated according to CPC1[45] and CPC2[46] methods. Enrichment with LR-related datasets was done by comparing the list of differentially expressed genes in RNAi-*ARES* (down and up) versus the different lists of differentially expressed genes present in the VisuaLRTC. Statistical enrichment was conducted with a hypergeometric test.[18,23,24,47] Only genes present on the ATH1 Affymetrix were kept for the enrichment analysis. Venn diagrams were drawn with jvenn.[48]

Splicing analysis was performed using SUPPA software (version 2.3,[49]) using AtRTD3 annotation.[50] Transcript accumulation was estimated using kallisto (version 0.46.1,[51]) with the following arguments -b 100--rf-stranded. Isoform and splicing event quantification and comparison were processed according to SUPPA's manual and the *p*-value was corrected using FDR.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest declared by authors.

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SUPPORTING INFORMATION

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