



Validation and expression analysis of candidate genes for adventitious rooting, in micro-cuttings of *Acca sellowiana* (Myrtaceae)

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Abstract

Acca sellowiana (Myrtaceae) is a multipurpose species with edible fruits and ornamental value, native to Uruguay and southern Brazil. Domestication of the species in Uruguay is incipient although in other countries, it is widely cultivated. It is an evergreen out-crossing shrub, pollinated by birds and bees. For this reason, it is necessary to develop vegetative propagation strategies such as stem cuttings to reproduce outstanding genotypes for conservation or breeding programs. Adventitious root (AR) formation in cuttings is regulated by environmental and endogenous factors. Among phytohormones, indole-butyric acid (IBA) is the most widely exogenous auxin used to improve rooting of cuttings. Most studies on AR formation at the molecular level use model species; however, the conservation of these mechanisms in non-model plants has been little studied, consequently the effects of different factors and their interactions in *A. sellowiana* are not well understood. The identification and expression analysis of genes known to be involved in the regulation of the process is an important step to elucidate the molecular mechanisms that regulate AR differentiation in *A. sellowiana* cuttings. In this study, we compared two genotypes with contrasting rooting ability, and we identified and characterized three genes that might regulate the onset of AR development in *A. sellowiana*: *AsPIN1*, *AsTIR1* and *AsSHR*. Their expression analysis showed that in the difficult-to-root genotype, *AsTIR1* increases strongly in response to exogenous IBA, shortly after induction treatment. Relative expression of *AsPIN1* and *AsSHR* also increases 24 h later. The biological significance of this gene expression pattern is discussed.

Keywords Adventitious rooting · Indole-butyric acid (IBA) · Vegetative propagation · Polar transport

Introduction

Acca sellowiana (Berg.) Burret is an evergreen shrub of the Myrtaceae family which is cultivated for its fruits and is also valued as an ornamental plant for its flowers and foliage. Extracts taken from fruits and leaves contain antioxidant, antimicrobial, and pharmacological activities (Vuotto et al. 2000; Bontempo et al. 2007; Mosbah et al. 2018; Tortora et al. 2019). Domestication and breeding of this species depend on the ability of elite plant materials to be propagated. However, adventitious root differentiation of cuttings

varies between 0 and 80% depending on the genotype set evaluated (Franzon et al. 2004; Guerra et al. 2012; Ross et al. 2017; Niella et al. 2018), and it is difficult to provide nurseries with mother plants of selected materials and particularly difficult-to-root genotypes.

It was hypothesized that these differences in AR formation among *A. sellowiana* genotypes are due to an earlier phase change in the difficult-to-root genotypes which can explain the loss of competence to form adventitious roots (Ross et al. 2021). In woody plants, the ability of cuttings to form adventitious roots declines with the age of donor plants (Wendling et al. 2014a; Aumond et al. 2017) and has been inversely linked with the xylogenesis program (de Almeida et al. 2012; Abarca et al. 2014; Vielba et al. 2016). Some genes of the GRAS family, such as *SCR* and *SHR*, are involved in the maturation-related decline of adventitious rooting (Pizarro and Díaz-Sala 2019). This might be explained by the different functions that the same gene regulatory network may play at different developmental stages

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of a cell type (De Lucas and Brady 2013). The lower rooting capacity associated with ageing of donor plants is little understood in woody plants (Aumond et al. 2017). Anatomy of stem cuttings in difficult-to-root genotypes of *A. sellowiana* shows an earlier development of the periderm associated with loss of competence to form adventitious roots (Ross et al. 2021). It is known that competence to form AR declines with phase change as a result of changes in auxin homeostasis (Rasmussen et al. 2014). The different rooting performance observed in genotypes with different rooting ability is mainly due to the regulation of endogenous active auxin accumulation in the AR source tissues after cutting excision, as well as differences in auxin sensitivity (Guan et al. 2015; Druege et al. 2016, 2019). This differential distribution of auxin seems to be a sufficient signal to trigger or modify the developmental program of a cell in model plants (Vanneste and Friml 2009; Negishi et al. 2011; Ruedell et al. 2015) although the mechanism has not been characterized in *A. sellowiana* specifically.

The fact that IAA is involved in the early events of AR formation has been well established (Blakesley 1994; Della Rovere et al. 2013) and the concentration of endogenous IAA is considered to play a central role in the control of AR initiation and development in various plant species (Ford et al. 2001; Kelen and Ozkan 2003; Davies 2010; Bellini et al. 2014; Pacurar et al. 2014; Wendling et al. 2015; Vilasboa et al. 2018; Gonin et al. 2019; Druege et al. 2019; de Almeida et al. 2020). The concentration of auxin in specific tissues is controlled by its biosynthesis, metabolism, and transport, and these processes are regulated by multiple mechanisms (Han et al. 2009). The differential accumulation of endogenous IAA in easy and difficult-to-root genotypes might be explained by the different expression of IAA biosynthesis and transport genes (de Almeida et al. 2015; Druege et al. 2019) as well as higher expression of repressors of auxin-responsive genes in hard to root species (Ruedell et al. 2015). AR formation depends on the early accumulation of IAA at the base of the cutting, via polar transport (Negishi et al. 2014; Pacurar et al. 2014; Druege et al. 2016). Quantification of indole 3 acetic acid (IAA) is not extensively used in AR studies because of its low concentration in tissues and the interference of other compounds in the analysis (Stuepp et al. 2016).

An alternative strategy to understand AR formation would be to identify and study the expression of genes involved in auxin homeostasis and root meristem patterning in the excised tissues of cuttings. Sensitivity to auxin is determined by the presence and affinity of auxin receptors. The auxin receptor *TRANSPORT INHIBITOR RESPONSE 1 (TIR1)* and its homologous gene *PagFBL1* increased their expression a few hours after auxin treatment (de Almeida et al. 2015; Shu et al. 2019). Once auxin sensitivity is established, other genes in the auxin response network which act

downstream in the AR signaling pathway are bound to activate (Teale et al. 2006; Pierre-Jerome et al. 2013; Wachsmann et al. 2015). Changes in gene expression are detected during the first hours after cutting excision in response to the stress caused by wounding mainly during the first 24 h (Druege et al. 2016).

Several genes are induced by exogenous auxin during the process of AR formation (Ludwig-Müller 2000; Guan et al. 2015; Druege et al. 2019). Auxin-induced AR formation in cuttings involves transcription factors of the GRAS family, particularly in the context of maturation of woody plants (Druege et al. 2016). Root meristem patterning and stem cell specification in response to auxin require of the GRAS family transcription factors such as *SHORTROOT (SHR)* (Blilou et al. 2005). These are also involved in the maturation-related decline of adventitious root formation in distantly related forest species, and the switch between the developmental programs of xylogenesis and AR formation in *A. thaliana* (Xuan et al. 2014; Abarca et al. 2014; Stevens et al. 2018; Pizarro and Díaz-Sala 2019). In other species, these genes are upregulated during AR formation and null mutants exhibit reduced AR formation (Druege et al. 2019). The expression of the auxin efflux carrier gene *PINFORMED 1 (PIN1)* increases in response to exogenous auxin; short auxin treatments activate the transcription of *PIN* genes in different tissues regulating its own distribution (Vanneste and Friml 2009; Fett-Neto et al. 2011).

AR formation in *Acca sellowiana* cuttings is strongly affected by genotype. Among several treatments used to induce rooting, difficult-to-root genotypes (NR) respond only to exogenous IBA and reach rooting levels similar to the easy-to-root genotypes (R) without exogenous hormone (Ross et al. 2017). Our hypothesis is that exogenous IBA improves AR formation in NR cuttings of *A. sellowiana* by modifying the expression of genes involved in auxin perception and homeostasis during the first stages of the process, leading to the acquisition of competence of some cells to form AR. In order to contribute to the understanding of AR formation and the causes of intraspecific variability specifically in *A. sellowiana*, the purpose of our study was to identify some of those genes in model species, validate them in *A. sellowiana* and study their expression pattern in response to exogenous IBA. In order to provide evidence that supports this interpretation of the differences among genotype, we selected a few but critical genes that have been shown to be involved in auxin homeostasis (*PINI*; *TIR1*) and root meristem patterning (*SHR*) in *E. grandis* and *A. thaliana*, to study their expression in micro-cuttings of two *A. sellowiana* genotypes with contrasting rooting ability.

Materials and methods

Plant material and culture conditions

Mother plants of *A. sellowiana* were provided by a local breeding program of the species (INIA-Facultad de Agronomía-MGAP; Uruguay). Two selected genotypes with contrasting rooting ability were grown in the greenhouse under controlled conditions, and treated periodically with fungicide (Benlate®, 0.2%) and Phostrogen® [NPK(MgO₃-SO₃): 14–10–27 (2.5–7.5)]. Genotypes were identified as R (easy-to-root) and NR (difficult-to-root), according to their rooting performance *ex vitro* (more than 60% and less than 20% rooting, respectively). To minimize the phenotypic differences in growth habit that might exist between genotypes, sprouts were collected from the basal branches of 4-year-old, vigorous healthy plants, in the same position in the branch. Apical segments (1.5–2.0 cm long) were surface-disinfected with 2% NaOCl for 15 min, washed three times with distilled water and introduced in vitro on WPM medium (Lloyd and McCown 1980) supplemented with MS vitamins (Murashige and Skoog 1962). Rooting of micro-cuttings was induced by adding IBA (9.8 μM) to the culture medium as previously described (Ross et al. 2017). Micro-shoots without IBA treatment were used as control. Cultures were incubated at 25 ± 2 °C, provided with a photon flux of 30 μmol m⁻² s⁻¹ and 16:8-h photoperiod.

Bioinformatics analysis and primer design of candidate genes and reference genes

Three candidate genes and three reference genes were chosen for further gene expression analysis. Candidate genes were selected based on literature for other species, in which they are known to be involved in auxin transport (*PIN1*),

auxin perception (*TIR1*), and root patterning (*SHR*). Reference genes were chosen among genes previously validated for their use during adventitious rooting in *Eucalyptus globulus* (*EF2*, *H2B*, *UBI*) (de Almeida et al. 2010). The primers used for reference genes were those validated for *E. globulus* by the Almeida et al. (2010), shown in Table 1.

For primer design, sequences of the three candidate genes in *A. thaliana* were taken from the GenBank database of the National Center for Biotechnological Information (NCBI) (<http://www.ncbi.nlm.nih.gov/genbank/>) and Plant Transcription Factor Database (PlantTFDB) (<http://plantfdb.gao-lab.org/>). A sequence similarity search within *E. grandis* genome was performed by BLAST analysis using the Phytozome12 platform (<https://phytozome.jgi.doe.gov/pz/portal.html>). The predicted amino acid sequences were compared and conserved amino acids are colored by the Jalview multiple alignment editor (Clamp et al. 2004).

Conserved regions were obtained by the alignment of the encoding sequences of both species (*A. thaliana* and *E. grandis*), using BioEdit® Sequence Alignment Editor software (Hall 1999). Primers were designed from these conserved regions using Primer3Plus (<https://primer3plus.com/>) and sequenced at Macrogen Inc. (Seoul, Korea). If differences in the sequences of both species were observed, the designed primers were biased towards the sequence corresponding to *E. grandis*. Two pairs of specific primers were designed for each candidate gene of interest (*PIN1*, *TIR1*, and *SHR*) based on the sequence alignment of conserved regions of the homologous genes in *A. thaliana* and *E. grandis* (Table 2).

Amplification and sequence analysis of candidate genes in *A. sellowiana*

To confirm whether the primers showed homology with genomic regions of *A. sellowiana*, genomic DNA was extracted from leaves of both genotypes of *A. sellowiana*,

Table 1 Primer sequences for reference genes (de Almeida et al. 2010)

Gene symbol	Forward primer (5'–3')	Reverse primer (5'–3')
<i>EF2</i>	GCGTCCCTCAGTGTGCTT	GGTCATCTGCTCCTTCAAGC
<i>UBI</i>	AGAAGGAATCGACCCTCCAC	CCTTGACGTTGTCAATGGTG
<i>H2B</i>	GAAGAAGCGGGTGAAGAAGA	GGCGAGTTTCTCGAAGATGT

Table 2 Primer sequences for the genes of interest designed using Primer3Plus software. Two pairs of primers were designed for each gene of interest *PIN1*, *SHR*, and *TIR1*

Gene symbol	Forward primer (5'–3')	Reverse primer (5'–3')
1- <i>PIN1</i>	CCTCATGGTCCAGATCGTC	CGAGTATATCTCAGCATTGGTTAGG
2- <i>PIN1</i>	ACGGGGTTCGACTTCTAC	TATACGAGGGCAGAGTAGACG
1- <i>SHR</i>	AAGACTTGCTCCTTCGAGTCCA	TCGGTCTTAACCGTCGGAAA
2- <i>SHR</i>	GCTGGCCTCCTACTTCTC	GCCTCGCAAATTTCTCCAT
1- <i>TIR1</i>	TCTTTAAGAATTTAAGGGTTCTTGT	AGCAATCCCAAAATCCAGA
2- <i>TIR1</i>	ACGCGAGCTGAGAGTGTTC	GATCGCATTGTCTCCAGCTT

using the cetyl-tri-methyl-ammonium bromide (CTAB) method (Doyle and Doyle 1987). DNA samples of *E. grandis* and *A. thaliana* were included as positive controls, using the same method. DNA quantity and quality were assessed by electrophoresis in agarose gel (0.8%) and spectrophotometry with a NanoDrop ND-1000 (Thermo Scientific[®]).

PCR amplification was performed in a 20- μ l reaction containing: 1 \times reaction buffer with 2-mM MgCl₂, 1-mM dNTPs, 0.5 μ M of each primer, 100-ng template DNA, and 0.5 U Taq DNA polymerase (Thermo Scientific[®]). The PCR program was as follows: 94 °C for 5 min, followed by 35 cycles of 94°C for 30 s, 52–58 °C for 30 s, and 72 °C for 40 s, using a Gene Touch Thermal Cycler (Bioer Technology[®]).

Amplification products were resolved by electrophoresis in an agarose gel (2%), stained with ethidium bromide and visualized under UV illumination. The size of the amplification products was estimated with 1 kb ladder (Thermo Scientific[®]) as molecular weight marker. Unique amplification products and their respective primers were sequenced at MacroGen Inc. (Seoul, Korea) and edited using FinchTV software (Geospiza Inc.[®]). Once edited, the final sequences were analyzed using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the UniProt database

(<https://www.uniprot.org/>). The sequences obtained were deposited to GenBank with accession numbers MZ130946-MZ13095.

Design of specific primers for qRT-PCR

Specific primers for qRT-PCR of target and reference genes were designed using Primer3Plus (Tables 3 and 4). More than one pair of primers for each gene of interest and reference gene was designed in order to obtain combinations with similar amplification efficiency for expression analysis. Target sequence size was confirmed on genomic DNA of *A. sellowiana* by PCR in 20- μ l reactions containing: 100-ng genomic DNA, 1 \times reaction buffer with 2-mM MgCl₂, 1-mM dNTPs, 0.5 μ M of each primer, and 0.5 U Taq DNA polymerase (Thermo Scientific[®]). The PCR program was as follows: 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 40 s, using a Gene Touch Thermal Cycler (Bioer Technology[®]). Amplification products were resolved in agarose gels (2%) with 1 \times TBE buffer solution and stained with ethidium bromide. The size of amplification products was estimated with 1 kb ladder (Thermo Scientific[®]) as molecular weight marker and visualized under UV illumination.

Table 3 Primer sequences of target genes used in qPCR analyses in *A. sellowiana*

Gene symbol	Forward primer (5'–3')	Reverse primer (5'–3')	Amplification size (bp)
<i>AsIPIN1(a)1</i>	CTCTGATGCTTTTCCTGTTCG	TTGGATGGAGACGATGGAC	94
<i>AsIPIN1(b)</i>	CTGATGCTTTTCCTGTTCGAG	GACTTGGATGGAGACGATGG	95
<i>As2PIN1(a)2</i>	CTTCCACTTCATCTCCTCCAAC	AAGAGGGTAATGGACCAGTCCG	149
<i>As2PIN1(b)</i>	CTTCCACTTCATCTCCTCCAAC	AGGGTAATGGACCAGTCCGAG	146
<i>As2SHR(a)</i>	CAGGGCTTGCTTAGTCGTATG	TGGAACCTGAGCACCATCTTC	115
<i>As2SHR(b)</i>	AAGATGGTGTCTCAAGTCCAG	CAATACGTGTGCTGATGTCCG	130
<i>As2TIR1(a)</i>	CATTGAACCACGTTGTCTCTG	CTTGACTGTTGGACAATGG	78
<i>As2TIR1(b)</i>	CCATTGTCCAACAGTGCAAG	GCCACAGAAAGCATCTCAAG	114

Table 4 Primer sequences of reference genes used in qPCR analyses in *A. sellowiana*

Gene symbol	Forward primer (5'–3')	Reverse primer (5'–3')	Amplification size (bp)
<i>H2B (a)</i>	AAGAAGCGGGTGAAGAAGAG	CGAAGATGTCGTTGATGAAGC	132
<i>H2B (b)</i>	AAGAGCGTGGAGACGTACAAG	CGAAGATGTCGTTGATGAAGC	117
<i>EF2 (a)</i>	TGATGTCCGATCCTTCTGG	TTCAAGCCCTTCTCTTACG	76
<i>EF2 (b)</i>	CAGTGTGTCTTCGATCACTGG	TTCAAGCCCTTCTCTTACG	106
<i>UBI (a)</i>	TCCCCTTGTTTCTTCGTCTC	CCTTGACGTTGTCAATGGTG	111
<i>UBI (b)</i>	CCTCCCCTTGTTTCTTCG	CCTTGACGTTGTCAATGGTG	114

RNA isolation and cDNA synthesis

Samples were collected 12 and 36 h after rooting induction treatment with IBA. Total RNA was extracted from bottom Sects. (10 mm) of three micro-cuttings per replicate, further purified using Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Invitrogen, Carlsbad, USA) to eliminate residual DNA, following the manufacturer's instructions. RNA concentration was determined using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific®). Micro-cuttings used for RNA extraction were discarded, so different repetitions were used for the rooting experiment.

Synthesis of cDNA was performed from 1 µg total RNA to the final 12 µl reaction mixture using MMLV-RT/SS reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). We tested different cDNA dilutions (1/5, 1/10, 1/25, 1/50, 1/100) to determine the best concentration to be used. The optimum dilution was chosen with a quantification cycle (Cq) between 18 and 22 cycles for all samples. A standard curve was generated for each gene of interest using a fivefold dilution series, which was used to calculate primer efficiencies.

Gene expression analysis in micro-cuttings

The relative expression levels of *TIR1*, *PINI*, and *SHR* at the base of micro-cuttings were determined by quantitative reverse transcription-PCR (RT-qPCR). The resulting data were analyzed by the Comparative Ct method (Livak and Schmittgen 2001). Results are presented as fold change in expression according to Eqs. 1 and 2.

$$\text{Foldchange} = 2^{-\Delta\Delta Ct} \quad (1)$$

$$\Delta\Delta Ct = \left[(Ct \text{ gene of interest} - Ct \text{ internal control})_{\text{treated sample}} - (Ct \text{ gene of interest} - Ct \text{ internal control})_{\text{untreated control}} \right] \quad (2)$$

First, the expression of these three genes in both genotypes without addition of exogenous auxin was compared to their expression in the R genotype at the beginning. We then compared the effect of exogenous IBA on the expression of the same genes, in both genotypes (R and NR) 12 and 36 h after the induction treatment.

PCR reactions were carried out using Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific) in a Line-Gene K Fluorescence Quantitative PCR Detection System (Bioer Technology) as follows: 5 min pre-denaturing at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 15 s. Three biological and two technical replicates of each sample were done. To confirm the

specificity of each PCR reaction, a heat dissociation curve (melting curve) was performed, from 60 °C to 90 °C, following the final PCR cycle. To study changes in gene expression at the onset of AR development, samples were harvested at two time points (12 and 36 h after excision).

The reference genes (RGs) used as internal control were Histone *H2B* and Elongation factor *EF2*, reported as reference genes for qPCR during in vitro adventitious rooting of *Eucalyptus globulus* (de Almeida et al. 2010) and validated for *A. sellowiana*. To check that the expression of these RGs was not influenced by the conditions of the experiment, the effect of genotype, experimental treatment, and time of sampling on their expression was validated by one-way ANOVA ($p=0.01$) using $2^{-\Delta Ct}$, where ΔCt ($Ct_{\text{sample}} - Ct_{\text{cal}}$) (Schmittgen and Zakrajsek 2000). Experimental data were analyzed by the Comparative Ct method (Livak and Schmittgen 2001), using the geometric mean of the RGs for normalization of the genes of interest expression (Vandesompele et al. 2002).

Experimental design and statistical analysis

The rooting experiment had a factorial design (2×2) with five replicates, where the factors were two genotypes (R and NR) and two levels of IBA (0 and 9.8 µM).

The gene expression experiment had a factorial design (2×2×2) with two genotypes (R and NR), two levels of IBA (0 and 9.8 µM) and two time points post treatment (12 and 36 h.). For each combination of factors, three biological and two technical replicates were performed.

Data were analyzed statistically by analysis of variance (ANOVA) and means were compared by Tukey's test, with a confidence level of $p \leq 0.05$, using Infostat® statistical software. Arcsine transformation was applied to response data before analysis. Data in figures are given as means ± SE.

Results

Adventitious root differentiation in response to exogenous auxin (IBA)

The effect of exogenous IBA on AR formation was observed after four weeks. In vitro rooting of NR micro-cuttings improved significantly (standard error 2.89, degrees of freedom 3) when exogenous IBA (9.8 µM) was added to the induction media ($p < 0.0001$). Rooting percentage increased 2.4-fold when compared to the control treatment in the absence of auxin (Fig. 1a) although the number of roots per explant was not significantly different ($p=0.4104$) (standard error 0.25, degrees of freedom 3) (Fig. 1b). No differences were detected in rooting percentage or root number of the R genotype after the addition of exogenous IBA (Fig. 1a

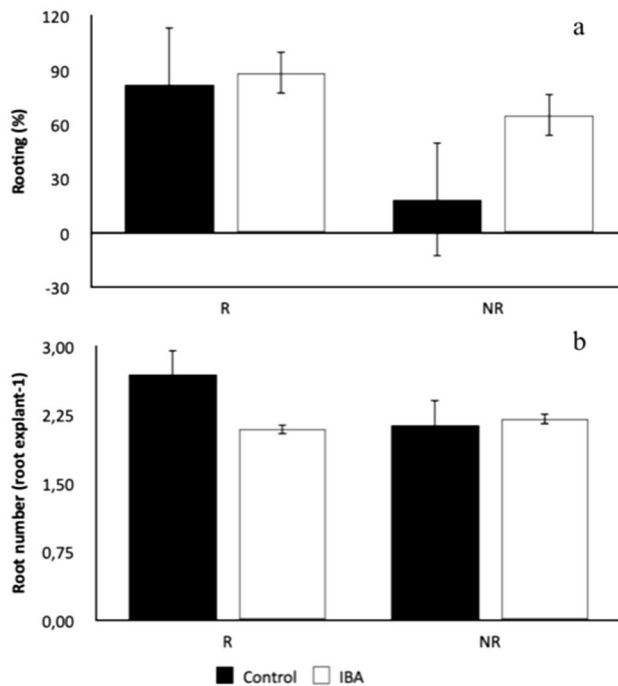


Fig. 1 Rooting performance in vitro of *A. sellowiana* genotypes with contrasting rooting ability, in response to exogenous IBA (9.8 μ M). R: easy-to-root genotype; NR: difficult-to-root genotype. **a** Rooting percentage; **b** Root number per rooted explant. Bars represent the mean values \pm SE

and b). New adventitious roots developed at the base of the micro-cuttings without callus formation in all cases (Fig. 2).

Amplification of target sequences in *A. sellowiana*

Unique amplification products of the expected size were obtained for the three genes of interest: *TIR1*, *SHR*, and *PIN1*. *SHR* and *TIR1* amplified with only one pair of the specific primers that we designed (Fig. 3a and b). *PIN1* amplified effectively with both pairs of specific primers designed. The resulting sequences (325 bp and 430 bp) partially overlapped and were assembled into a unique sequence (Fig. 3c). The three sequences obtained showed high identity with their homologous genes in *E. grandis* (>90%) and *A. thaliana* (>75%), according to NCBI and Uniprot databases respectively (Table 5).

The reference genes chosen for this study resulted in unique amplification products of the expected size in *A. sellowiana*: *EF2*, *H2B*, and *UBI*. The resulting sequences showed high identity with their homologous genes in *E. grandis* (>90%) and *A. thaliana* (\geq 69%), according to NCBI and Uniprot databases respectively (Table 5, Fig. 4). The predicted amino acidic sequences also showed high

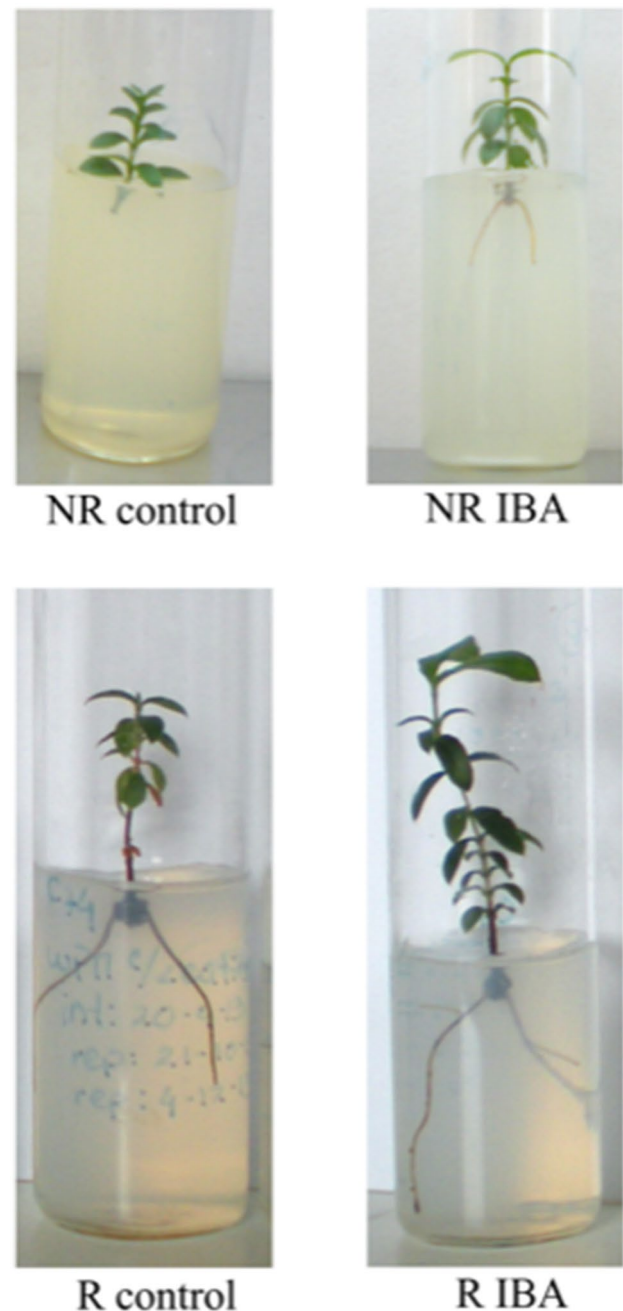


Fig. 2 Examples of root formation in samples of both studied genotypes with and without exogenous IBA. R: easy-to-root genotype; NR: difficult-to-root genotype

identity with the corresponding proteins in *Eucalyptus* and *A. thaliana* (Table 6).

Primers for qPCR

Two pairs of specific primers for qRT-PCR for the genes of interest *TIR1*, *SHR* and the reference gene *H2B* were designed (shown in Fig. 2). However, for the gene of

Table 5 Size of the cDNA sequences obtained in *A. sellowiana* for the genes of interest (*AsPIN1*, *AsSHR*, and *AsTIR1*) and reference genes (*AsEF2*, *AsH2B*, and *AsUBI*), and the corresponding identity with their homologous genes in *E. grandis* and *A. thaliana* according to NCBI and Uniprot databases

Gene symbol	Size (bp)	NCBI identity (%) with <i>E. grandis</i>	NCBI reference number	Uniprot identity (%) with <i>A. thaliana</i>	Uniprot reference number	Domain
<i>AsPIN1</i>	672	94	XM_010035442.1	76.4	Q9C6B8-1	Transmembrane
<i>AsSHR</i>	426	92	XM_010029789.1	75.3	Q9SZF7	GRAS
<i>AsTIR1</i>	521	95	XM_010039868.1	84.8	Q570C0-1	AMN
<i>AsEF2</i>	135	96	XM_010062544.1	69	Q9ASR1	
<i>AsH2B</i>	152	97	XM_010043098.1	94	Q9SI96	
<i>AsUBI</i>	129	98	XR_726060.1	85	Q42009	

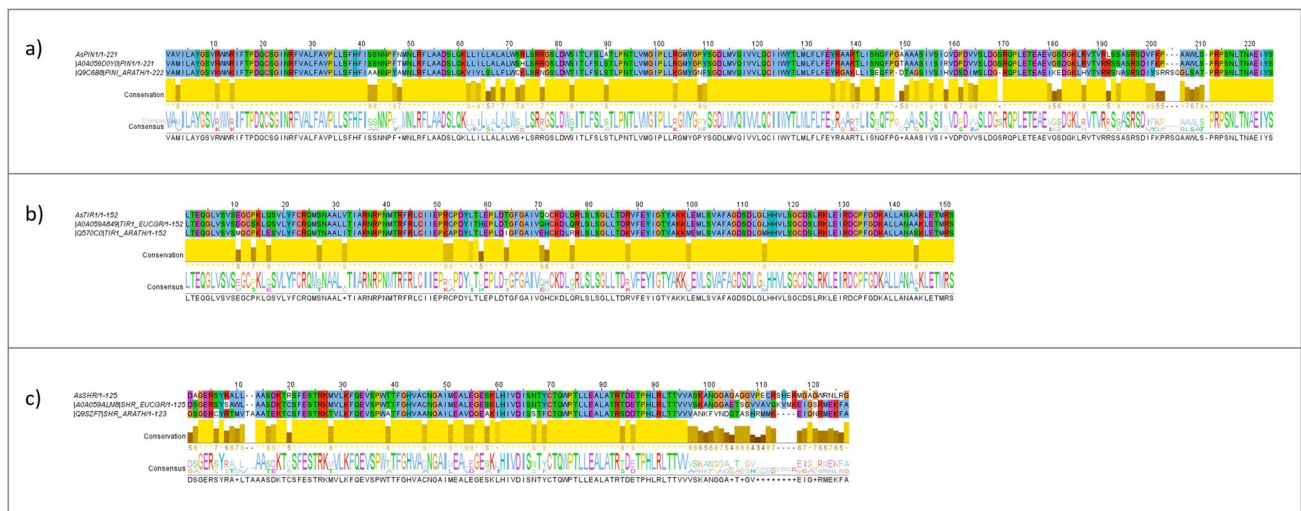


Fig. 4 Alignment of the amino acid sequences of **a** *AsPIN1*, **b** *AsTIR1* and **c** *AsSHR* with homologous sequences from *E. grandis* and *A. thaliana*, using Jalview. Most conserved regions are indicated with yellow bars below the alignment. A consensus sequence is shown in the last line

Table 6 Identity of the amino acid sequences obtained in *A. sellowiana* for the genes of interest (*AsPIN1*, *AsSHR*, and *AsTIR1*) and reference genes (*AsEF2*, *AsH2B*, and *AsUBI*), with their homologous genes in *E. grandis* and *A. thaliana* according to Uniprot database

	<i>Eucalyptus</i> (Uniprot reference number)	% identity (aa)	<i>A. thaliana</i> (Uniprot reference number)	% identity (aa)
<i>AsPIN1</i>	A0A059D0Y0	95	Q9C6B8	77
<i>AsSHR</i>	A0A059ALM8	91	Q9SZF7	65
<i>AsTIR1</i>	A0A059A849	97	Q570C0	91
<i>AsEF2</i>	A0A059BNB6	81	Q9ASR1	70
<i>AsH2B</i>	A0A059AF50	96	Q9SI96	94
<i>AsUBI</i>	A0A059DDH9	77	Q42009	77

Analysis of gene expression in micro-cuttings

Relative expression at the onset of AR induction was measured in micro-cuttings of *A. sellowiana* by RT-qPCR using the geometric mean of two reference genes (*H2B* and *EF2*). *AsTIR1*, *AsPIN1*, and *AsSHR* transcripts were induced in response to exogenous auxin in the difficult-to-root genotype during the early steps of AR formation. First, we examined the expression of these genes in

both genotypes (R and NR) without IBA, relative to their expression in the R genotype without IBA 12 h after the onset of the experiment. When no exogenous IBA was added to the medium, the expression of these three genes was lower in the NR genotype than in the R genotype ($p < 0.0001$) throughout the period under study (Fig. 5). Without the addition of exogenous IBA, the R genotype showed an increase in the expression of the three genes 36 h after the beginning of the experiment ($p < 0.0001$).

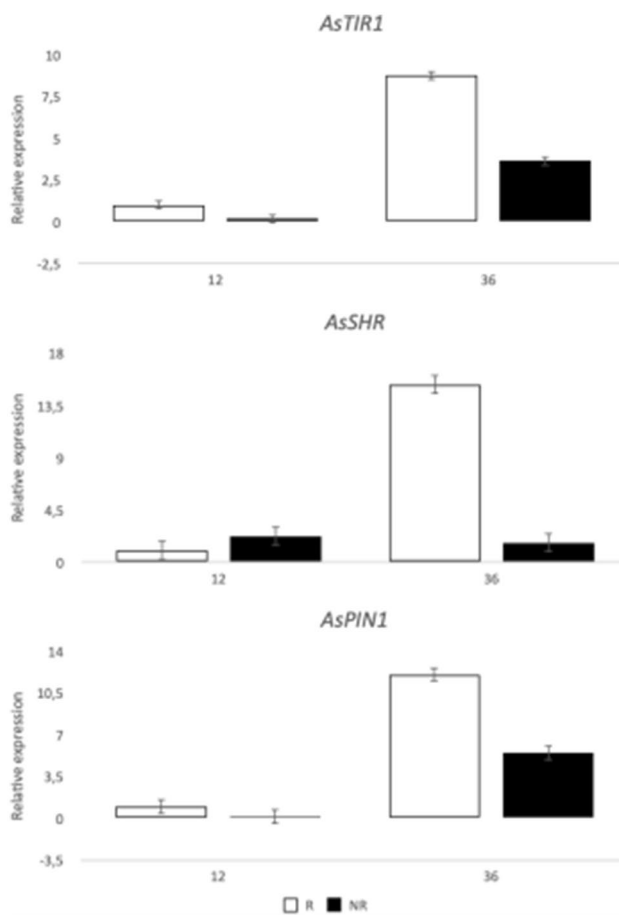


Fig. 5 Expression of *AsTIR1*, *AsSHR* and *AsPIN1* without exogenous IBA in the R and NR genotypes of *A. sellowiana* micro-cuttings relative to their expression 12 h after the beginning of the experiment. R: easy-to-root genotype; NR: difficult-to-root genotype. Data were analyzed by the Comparative Ct method ($2^{-\Delta\Delta Cq}$) using the geometric mean of the reference genes (*H2B* and *EF2*). Bars represent the mean relative expression \pm SE of three independent biological replicates and two technical replicates

In the NR genotype, on the other hand, *AsTIR* and *AsPIN* also increased their expression ($p < 0.0001$) but in a much lower magnitude. Data were analyzed by the Comparative Ct method ($2^{-\Delta\Delta Cq}$) using the geometric mean of the reference genes (*H2B* and *EF2*). The coefficient of variation between technical replicates was < 1 for the three genes of interest. $\Delta\Delta Ct = [(Ct \text{ gene of interest} - Ct \text{ internal control})_{NR \text{ genotype}} - (Ct \text{ gene of interest} - Ct \text{ internal control})_{R \text{ genotype}}]$

Next, we compared the expression of these genes between treated ($0.98 \mu\text{M}$ IBA) and untreated ($0 \mu\text{M}$ IBA) samples and found that the behavior of the R and NR genotypes was clearly different in response to the exogenous auxin. The relative expression of *TIR1*, *SHR*, and *PIN1* increased in

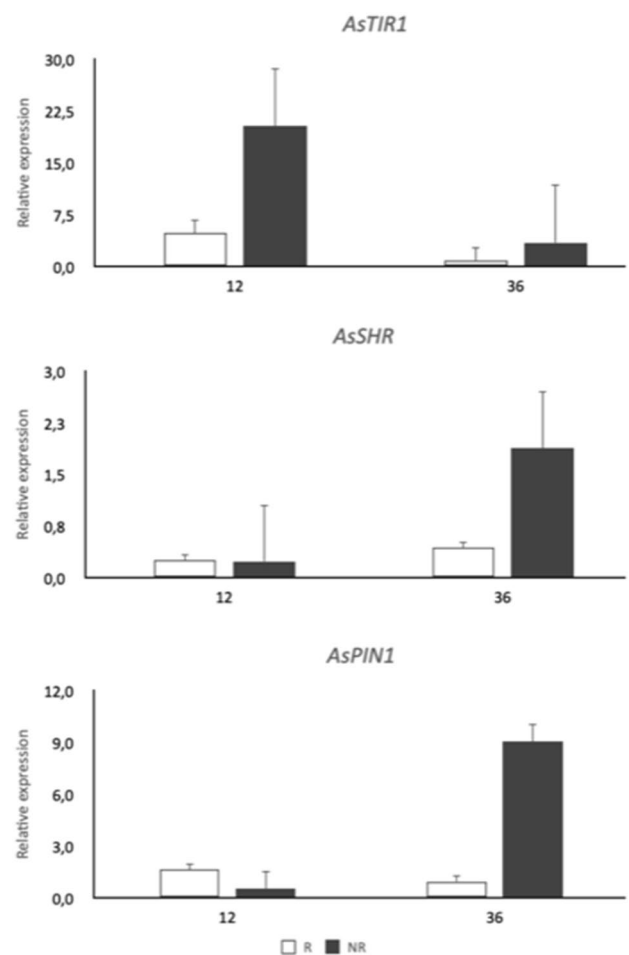


Fig. 6 Relative expression of *AsTIR1*, *AsSHR* and *AsPIN1* in treated ($0.98 \mu\text{M}$ IBA) vs. untreated ($0 \mu\text{M}$ IBA) samples of two genotypes of *A. sellowiana* micro-cuttings with contrasting rooting ability, 12 and 36 h after induction treatment with IBA ($9.8 \mu\text{M}$). R: easy-to-root genotype, NR: difficult-to-root genotype. Data were analyzed by the Comparative Ct method ($2^{-\Delta\Delta Cq}$) using the geometric mean of the reference genes (*H2B* and *EF2*). Bars represent the mean relative expression \pm SE of three independent biological replicates and two technical replicates. $\Delta\Delta Ct = [(Ct \text{ gene of interest} - Ct \text{ internal control})_{\text{sample with IBA}} - (Ct \text{ gene of interest} - Ct \text{ internal control})_{\text{sample without IBA}}]$

response to IBA, but a much greater response was observed in the NR genotype in the treated vs untreated samples. An increase in the expression of the auxin receptor *TIR1* was induced 12 h after the treatment with exogenous IBA ($p = 0.0111$), while the expression of the auxin efflux carrier *PIN1* and the transcription factor *SHR* increased in the NR genotype 36 h after treatment with exogenous IBA relative to their expression in the untreated sample ($p = 0.0157$ and 0.0189 respectively) (Fig. 6). The relative expression of *TIR1* and *SHR* increased in the R genotype in response to IBA but to a lesser extent ($p = 0.0455$ and 0.0189 respectively), while

changes in relative expression of *PIN1* was not significant ($p=0.1216$).

Discussion

When a well-curated genome of the species under study is not available, the genome of a related species can be used as reference; however, the exact sequence and the genomic location of the genes of interest are unknown. Amplification and sequence comparison can provide evidence to hypothesize that the transcripts that are being quantified are homologous to the genes reported in model species. In this study, we successfully amplified regions of genes which have been shown to be involved at different stages during the onset of AR formation based on published genomes of model species. We showed that the genomic sequences obtained from *A. sellowiana* are consistent with the target genes and observed a pattern of expression that can be interpreted in the light of the current general understanding of AR formation and previously published hypotheses about intraspecific variability in *A. sellowiana* for this trait.

Unique amplification products of the expected size were obtained for three genes of interest: *TIR1*, *SHR*, and *PIN1*. Sequence analysis of the obtained PCR fragments showed that they presented high sequence similarity with the candidate genes *PIN1*, *SHR*, and *TIR1*. For the three genes, identity with *E. grandis* and with *A. thaliana* was high (more than 90% and 70% respectively). Furthermore, the protein sequences resulting from the translation of the isolated gene fragments present, although partially, domains characteristic of the PIN1, SHR, and TIR1 proteins. AsPIN1 sequence includes part of a transmembrane domain, a domain present in PIN1 from *Arabidopsis* (Gälweiler et al., 1998), while AsSHR sequence contains a significant portion of the GRAS domain (Pysh et al. 1999; Helariutta et al. 2000). When analyzing the results in AsTIR1, the isolated fragment presents a partial AMN domain, which corresponds to 4 complete LRRs (Leucine-Rich Repeats). This AMN domain contains 16 LRRs in *A. thaliana*. Although the length of the isolated fragment did not include the FBox domain, which is the other descriptive feature for the *TIR1* gene (Ruegger et al., 1998), the isolated fragment shows 95% and 84.6% identity with the homologous fragments of *E. grandis* and *A. thaliana* respectively. According to these results, *TIR1*, *PIN1*, and *SHR* genes are present in the *A. sellowiana* genome, and their sequences show a similarity of more than 90% with the respective *E. grandis* genes in the region of the gene that was isolated.

Our expression analysis indicates that *AsTIR1*, *AsPIN1*, and *AsSHR* have different levels of expression in R and NR genotypes when there is no hormonal treatment. The level of expression of these three genes in the difficult-to-root

genotype 36 h after the beginning of the experiment was always lower than in the easy-to-root genotype. *AsTIR1*, *AsPIN1*, and *AsSHR* transcripts are induced in response to exogenous IBA in stem cuttings of difficult-to-root genotypes of *A. sellowiana*, during the early steps of AR formation. The R genotype already had a high expression without IBA, so the response to exogenous auxin is not as strong as the increase in relative expression of these genes in the NR genotype. This result is in agreement with the rooting behavior itself; the R genotype roots well without the addition of IBA while the NR genotype significantly improves rooting levels when the exogenous auxin is added to the culture media. This may be explained by the presence of an endogenous higher level of auxin in the R genotype. Among various conditions previously evaluated to induce rooting, exogenous IBA was the only treatment that improved the AR capacity of this genotype to levels that are similar to the easy-to-root genotype (Ross et al. 2017). However, the R genotype does not improve rooting in response to exogenous IBA, and the expression of these genes remains stable. Our data show that the expression of these genes was already much higher in the untreated R genotype, and the addition of exogenous IBA had a very small effect on this genotype. In the difficult-to-root genotype, on the other hand, the relative expression of *AsTIR1* increases strongly in response to exogenous IBA, shortly after the induction treatment. In *Populus*, the expression of *PagFBL1* (homolog of *TIR1* in *Arabidopsis*) was similar to the distribution pattern of auxin during AR formation, with a high expression in the cambium and secondary phloem during the induction and initiation phases that decreased in the emerging primordia (Shu et al. 2019). Although our expression analysis was not focused on specific tissues, neo-formation of adventitious root meristems in *A. sellowiana* takes place outside the cambial ring of the stem, in the secondary phloem (Ross et al. 2021). Thus, it is possible that the increase in the expression of *AsTIR1* that we found is concentrated in this tissue and it would be interesting to explore how the expression varies in different tissues of the stem cutting. The relative expression of *AsPIN1* and *AsSHR* also increases, but 24 h later. The effect of IBA as a rooting agent for difficult-to-root materials of this species can thus be related to the increase in expression of at least these genes. These findings are congruent with our previous results and support our hypothesis of an earlier phase change from juvenile to mature of the NR genotype of *A. sellowiana*. We found that loss of competence to form AR was associated with an earlier phase change, evidenced by the differentiation of a periderm in the NR genotype (Ross et al. 2021).

As plants age, there is a lower expression of the main auxin receptor (TIR1) that explains the loss of sensitivity to auxin (Aumond et al. 2017). The improvement of rooting ability of the NR genotype of *A. sellowiana* when exogenous

IBA was added may have resulted from the increased expression of the auxin receptor *AsTIR1*. This increase in the sensibility to auxin may have led to a further modification in the expression of other genes that act downstream auxin perception and that play essential roles in the rooting of cuttings. Among many other genes involved in AR differentiation, the increase in relative expression of *AsPIN1* and *AsSHR* in NR genotype is congruent with the observed improvement of rooting ability after exogenous IBA treatment. As a consequence of the concerted action of these genes which showed higher expression in response to IBA, the NR genotype improved the rooting performance, reaching levels similar to the R genotype with or without exogenous IBA. Similar changes in gene expression in response to exogenous auxin have been reported in other species of Myrtaceae (Fett-Neto et al. 2011; de Almeida et al. 2015). The relative amount of mRNA of *SHR* and other transcription factors of the GRAS family has been observed to be significantly reduced in adult tissues that have lost the capacity to develop AR. The expression of *SHR* during AR formation is affected by age, auxin level and developmental stage of the cells (Abarca et al. 2014). In *Arabidopsis*, the same cells that are reactivated by auxin to differentiate ARs, are also able to initiate xylogenesis, with *SHR* among other transcription factors controlling the switch between the programs (Ricci et al. 2016).

The kind of changes in gene expression that we observed in the different genotypes resembles those observed in different stages during AR development in other species of the Myrtaceae family. Studies in other species of the Myrtaceae with poor AR development also show lower expression of auxin receptors. Loss of AR ability in *E. globulus* micro-cuttings has been explained by a combination of lower expression of auxin receptors (*TIR1*, *ABP1*) and higher expression level for auxin repressors (*IAA12*, *TPL*, *ARR1*). Furthermore, the expression of genes related to auxin synthesis (*TAA1*, *YUC3*) and transport (*PINI*, *AUX1*) was found to diverge between stages of development and auxin treatment (Vilasboa et al. 2018).

Although the regulatory gene network that controls AR formation in *A. sellowiana* or its specific differences from better known species is far from being completely understood, we found evidence that supports the hypothesis that earlier phase change underlies the reduced competence to differentiate new roots in the NR genotypes. Several techniques have been used to delay maturation of juvenile plants or reverse the physiological status of adult plants in other species (Wendling et al. 2014b; Benedini et al. 2015; Stuepp et al. 2016; Bisognin et al. 2017, 2018); among these, epicormic shoots induced by pruning, coppicing, or girdling of adult trees could be evaluated as a source of rejuvenated material for stem cuttings of the NR genotypes of *A. sellowiana*.

Conclusions

We identified and characterized three genes that are induced by IBA and are likely related to AR development in *A. sellowiana* micro-cuttings: *AsPIN1*, *AsTIR1*, and *AsSHR*. The results of the expression analysis showed that in the difficult-to-root genotype, *AsTIR1* increases strongly in response to exogenous IBA, shortly after induction treatment improving sensibility to auxin of the cells. Relative expression of *AsPIN1* and *AsSHR* also increases, but 24 h later.

These results indicate that *AsTIR1*, *AsPIN1*, and *AsSHR* transcripts are induced during the early steps of AR formation in response to exogenous IBA in stem cuttings of the difficult-to-root genotype of *A. sellowiana*, improving AR formation. This behavior is similar to that of mature tissues studied in other species.

Our results show that cloning of *A. sellowiana* by stem cuttings requires physiologically juvenile or rejuvenated material and that different genotypes may require different treatments to produce competent cuttings.

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Author contribution statement Conceptualization: SR and PS; Methodology: SR, SR-D; JPS and GP; Formal analysis and investigation: SR; Writing—original draft preparation: SR; Writing—review and editing: SR, PS and OB; Funding acquisition: SR and PS; Project administration: SR; Supervision: PS.

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Data availability The datasets generated and analyzed during the current study are available in the GenBank repository at <https://www.ncbi.nlm.nih.gov/genbank/>, reference number MZ130946-MZ13095.

Declarations

Conflict of interest The authors declare they have no financial interests. The authors have no competing interests to declare that are relevant to the content of this article.

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