Transcriptomic analyses provide insight into adventitious root formation of *Euryodendron excelsum* H. T. Chang during ex vitro rooting

Yuping Xiong1,2 · Shuangyan Chen3,4 · Zhenpeng Wei5 · Xiaohong Chen1,2 · Beiyi Guo1,2 · Ting Zhang1,2 · Yuying Yin1,2 · Xincheng Yu5 · Jinhui Pang1,2 · Meiyun Niu1,2 · Xinhua Zhang1 · Yuan Li1 · Kunlin Wu1 · Lin Fang1 · Jaime A. Teixeira da Silva6 · Guohua Ma1 · Songjun Zeng1

Received: 13 October 2021 / Accepted: 29 December 2021 / Published online: 21 January 2022
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Abstract

*Euryodendron excelsum* H. T. Chang, a critically endangered species endemic to China, is a source of valuable material for the furniture and construction industries. However, this species has some challenges associated with rooting during in vitro propagation that have yet to be resolved. In this study, we optimized rooting and conducted a transcriptomic analysis to appreciate its molecular mechanism, thereby promoting the practical application of in vitro propagation of *E. excelsum*, and providing technical support for the ecological protection of this rare and endangered species. Results showed that ex vitro rooting performed the highest rooting percentage with 98.33% at 25 days. During ex vitro rooting, there was a wide fluctuation of endogenous levels of indole-3-acetic acid (IAA) and hydrogen peroxide (H2O2) at the stage of root primordia formation. Transcriptome analysis revealed multiple differentially expressed genes (DEGs) involved in adventitious root (AR) development. DEGs involved in plant hormone signal transduction, such as genes encoding auxin-induced protein, auxin-responsive protein, and auxin transporter-like protein, and in response to H2O2, oxidative stress, abiotic and biotic stimuli were significantly up- or down-regulated by ex vitro treatment with 1 mM indole-3-butyric acid (IBA). Our results indicate that ex vitro rooting is an effective method to induce AR from *E. excelsum* plantlets during micropropagation. DEGs involved in the plant hormone signal transduction pathway played a crucial role in AR formation. H2O2, produced by environmental stimulation, might be related to AR induction as a result of the synergistic action with IBA, ultimately regulating the level of endogenous IAA.

Key message

Under ex vitro rooting, a synergistic action between H2O2 produced by environmental stimulation and IBA played crucial role in the regulation of AR formation from *E. excelsum* plantlets during micropropagation.

Keywords *Euryodendron excelsum* H. T. Chang · Transcriptome · Adventitious roots · Hydrogen peroxide

Introduction

Adventitious root (AR) system is one of plant roots systems that arises from parts of the plant rather than from the roots of the embryo (Barlow 1986). ARs derived from non-root tissues, are the main path by which new plantlets root in vegetative propagation, and usually generated during normal development or stress conditions (Steffens and Rasmussen 2016). In vitro propagation via tissue culture has become an important technology in plant conservation strategies given its advantages, such as high propagation coefficient,
freedom from restrictions imposed by season, especially for rare and endangered species (Bhardwaj et al. 2018; Khater and Benbouza 2019; Rameshkumar et al. 2017). However, in some in vitro propagation systems, plants may display rooting-recalcitrance problems, for example by Juniperus thurifera L. (Khater and Benbouza 2019), Zeyheria montana Mart. (Cardoso and Teixeira da Silva 2013), Elegia capensis (Burm. f.) Schelpe (Verstraeten and Geelen 2015), and Cariniana legalis (Lerin et al. 2021). Rooting-related problems limit the application of in vitro propagation for plant breeding and conservation efforts. Therefore, AR formation during plant in vitro culture is a top research objective for plant asexual propagation breeders.

Plant growth regulators (PGRs) are commonly AR inducers used in in vitro culture, such as 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), but these tend to show species- and concentration-dependent AR induction efficiency. For Laburnum anagyroides Medic., a low concentration of IAA, IBA or NAA induced AR normally, but high concentrations induced callus formation in shoot tips and subsequently, plant death (Timofeeva et al. 2014). 0.25 or 0.5 μg/L of NAA promoted rooting during in vitro culture of Cornus alba L., while IBA had an adverse effect on root growth and even inhibited AR induction at 1.0 μg/L (Ilczuk and Jacygrad 2016). Moreover, in vitro rooting and ex vitro rooting also display some differences in AR formation. In vitro rooting to induce AR is always performed under aseptic conditions (Barpet et al. 2014; Guo et al. 2019; Nourissier and Monteuuius 2008). In contrast, ex vitro rooting employs unrooted plantlets that are removed from aseptic in vitro conditions culture to induce AR in an open environment (Revathi et al. 2018; Shekhawat and Manokari 2016). Although the two culture methods are affected by various factors, ex vitro rooting can enhance rooting percentage and survival during plant acclimatization, and reducing limiting factors in micropropagation (Benmahoul et al. 2012; Yan et al. 2010). For example, Ceratonia siliqua L. plantlets treated with 4.8 μM IBA displayed a 46.3% rooting response, forming a fragile root system when rooted in vitro whereas the induction of AR from ex vitro shoots treated with 14.4 μM IBA showed significantly higher rooting percentage (91.7%), and a normal morphological appearance, and were successfully acclimatized, showing more than 90% survival (Lozzi et al. 2019).

The mechanism of AR induction involves various key genes, proteins, and pathways (Chen et al. 2020a; Qi et al. 2020; Stevens et al. 2018). Most PGRs promote AR development by regulating the level of endogenous IAA, thus genes and pathways related to the biosynthesis and transport of IAA are considered to play a significant role in AR formation (Lakehal and Bellini 2019). Transcriptome sequencing revealed that candidate genes involved in AR formation of Mangifera indica L. cv. Zihua cotyledon segments were predicted to encode polar auxin transport carriers, auxin-regulated proteins and cell wall remodeling enzymes (Li et al. 2017). In Arabidopsis thaliana, IBA induced AR formation in thin cell layers by conversion into IAA involving nitric oxide activity, and by positive action on IAA transport and biosynthesis (Fattorini et al. 2017). Genes related to the synthesis, transport, metabolism and recognition of plant hormone were involved in the in vitro induction and elongation of ARs in Populus euramerica (Zhang et al. 2019b). However, knowledge of the molecular aspects of adventitious rooting in plants, especially in woody species that are recalcitrant to rooting, remains scanty. Understanding the mechanism of AR formation is of great importance to strategize plant breeding and conservation efforts to maximize the marketable yield and research value, especially of rare and endangered plants.

Euryodendron excelsum H. T. Chang, a monotypic genus endemic to China, is fine-textured and colorful, making it a source of valuable material for the furniture and construction industries (Chang 1963). However, mainly as a result of habitat destruction and deforestation caused by human activity, only a single population of E. excelsum can now be found at Bajia Zhen, Yangchun County, Guangdong Province, in southern China (Shen et al. 2008; Ye et al. 2002). E. excelsum is naturally propagated only by seeds, but seed germination and seedling growth toward adulthood are fragile stages that limit natural recruitment and regeneration (Shen et al. 2009, Wang et al. 2002). Based on the categorization of the International Union for Conservation of Nature and Natural Resources (IUCN), E. excelsum has been listed as a critically endangered plant since 1998, and continues to maintain this status and faces a high risk of extinction, implying that some strategies were put forward for the conservation of E. excelsum populations (Barstow 2020).

In our previous study, a micropropagation system for E. excelsum was established by in vitro culture. When treated with either IBA or NAA, in vitro E. excelsum showed lower rooting percentage in agarized woody plant medium (WPM) (Lloyd and McCown 1980) than in agar-free vermiculite-based WPM after culture for 2 months, and callus formed at the base of stems in these media, hampering the successful transplantation of plantlets (Chen et al. 2020b). We inferred from that there may be other factors that can stimulate AR formation in E. excelsum when cultured in vitro, or that enhance the induction efficiency of PGRs. Thus, the objectives of this study were to improve the micropropagation system of E. excelsum by optimizing the AR induction conditions, and to reveal the key influencing factors and related genes and processes underlying AR formation by transcriptomic analysis. By better elucidating the mechanism of AR formation of E. excelsum in vitro, research on biological
conservation and genetic engineering of *E. excelsum* can be promoted and advanced.

**Materials and methods**

**Culture of plantlets**

The basic micropropagation system for *E. excelsum* that was previously established (Chen et al. 2020b), was employed in this study. In vitro plantlets were maintained and propagated on WPM supplemented with 4.44 μM BA (Solarbio, Beijing, China) and 0.53 μM NAA (Macklin, Shanghai, China). Single shoots with more than four leaves and two nodes cut from multiple shoots were inoculated on PGR-free WPM for 30 days. During this period, AR was not induced. All media contained 20 g/L sucrose and 0.5% (w/v) agar, and pH was adjusted to 5.8–6.0, then autoclaved at 116 °C for 30 min. Culture jars (140 cm high; 90 cm diameter; 550 mL) were placed in an air-conditioned culture room at 25 ± 2 °C with a 12-h photoperiod under 100 μM m⁻² s⁻¹ fluorescent light (Philips, Tianjing, China) and 50–70% relative humidity.

**Adventitious root induction**

For the in vitro rooting treatment, 2 mm of the base of single shoots cultured on PGR-free WPM for 30 days were cut and trimmed shoots were inoculated on WPM supplemented with 0, 0.05, 0.5, and 5 μM NAA, IBA or IAA (Macklin). Shoots in the control group were inoculated on PGR/auxin-free WPM. Ten shoots were placed in each jar, and four jars were prepared for each treatment. Three replicates were performed for each treatment (n = 12 jars; 120 shoots in total).

For the ex vitro rooting treatment, single shoots cultured on PGR-free WPM for 30 days were removed from culture jars, and about 2 mm was trimmed from the base. Trimmed shoots were treated with 0, 1, 2, and 3 mM NAA, IBA or IAA for 10 min, then transferred to plates (5 cm in height; 27 cm in width; 47 cm in length) for raising seedlings supplemented with vermiculite and perlite (v/v, 1:1). Trimmed shoots cultured on PGR/auxin-free WPM served as the control. Forty shoots were planted in each plate, and three replicate plates were prepared for each treatment (n = 3 plates; 120 shoots in total).

Rooting percentage as well as average root number and root length were calculated for each treatment. After one-way analysis of variance (ANOVA), treatment means were compared by Duncan’s multiple range test (DMRT) in SPSS Statistics version 20.0 (IBM, New York, USA) and were considered to be significant difference between the designated treatments at *P* < 0.05.

**Histological analysis**

The base of shoots (0.5–1.0 cm) was collected at 0, 2, 4, 6, 8, 10 and 12 days after the optimum treatment method under ex vitro rooting and fixed for 24 h in formalin/acetic acid/alcohol at 25 ± 2 °C. At least 15 bases were collected for each time point. Fixed material was dehydrated in a 70–100% alcohol dehydration series followed by infiltration with molten paraffin (Macklin), and embedded in paraffin wax. Sections (8–10 μm thick) were made with a rotary microtome (KEDEE, Zhejiang, China) and stained in 0.02–0.05% toluidine blue (Macklin). Sections were viewed with a Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan) and micrographs were captured using a HQimage C630 digital camera (Hengqiao, Hangzhou, China).

**Determination of endogenous IAA and hydrogen peroxide (H₂O₂) content**

To analyze IAA and H₂O₂ content, the same method and growth conditions were employed as for histological analysis. Material was stored at −80 °C. Three biological replicates of 10 cut stem bases were harvested as 0.1 g fresh weight (FW) to assess endogenous IAA and H₂O₂ content, according to the instructions of an IAA Enzyme Linked Immunosorbent Assay kit (Dogesce, Beijing, China) (Zhang et al. 2017) and Hydrogen Peroxide Assay kit (Solarbio, Beijing, China) (Wang et al. 2019). After one-way ANOVA, treatment means were assessed by DMRT in SPSS Statistics version 20.0 and were considered to be significantly different between the designated treatments at *P* < 0.05.

**Isolation of RNA and cDNA library construction**

The same samples used to analyze IAA and H₂O₂ content were employed for RNA-seq analysis. Samples collected from 0, 2, 4, 6, 8, 10, and 12 days were marked as ER0, ER2, ER4, ER6, ER8, ER10, ER12, respectively, and stored at −80 °C. The Column Plant RNAOUT Extraction kit (Tianz, Beijing, China) was used to isolate total RNA from each sample, using the methods suggested by the manufacturer. The concentration and quality of all RNA samples was examined by agarose electrophoresis on an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Sequencing libraries were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Magnetic beads with oligo (dT) were used to purify mRNA, which was fragmented into short fragments (200–300 bp). Cleaved mRNA fragments were primed with a random hexamer primer for first-strand and second-strand cDNA
synthesis. After purification, end repair, and ligation to sequencing adapters, 21 cDNA libraries of three biological replicates for each treatment were prepared and sequenced using the Illumina Novaseq 6000 platform by Personal Biotechnology Co., Ltd. (Shanghai, China).

Sequencing and functional annotation

The raw reads were filtered to include only those with a median Phred quality score of R20 and trimmed with Cutadapt v2.7 to remove adapter sequences. Clean reads were obtained from the raw reads after adapter sequences and low-quality reads discarded. The de novo assembly of high-quality reads was performed by Trinity software with default parameters (version 2.5.1, https://github.com/trinityrnaseq/trinityrnaseq/wiki). The quality of the assembled transcript was examined by BUSCO v5.2.2. Assembled transcripts were aligned to NCBI non-redundant protein sequences (NR, http://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/), Gene Ontology (GO, https://www.blast2go.com/), Kyoto Encyclopedia of Genes and Genomes (KEGG, KAAS, http://kobas.cbi.pku.edu.cn/), evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG, http://egg-nog.embl.de/version_3.0), Swiss-Prot (http://www.uniprot.org/help/uniprotkb) and Pfam (http://pfam.xfam.org/) using BLASTX with a significance threshold of $E \leq 10^{-5}$.

Identification and functional annotation of differentially expressed genes (DEGs)

Gene expression level was calculated with the fragments per kilobase per transcript per million mapped reads method (FPKM). In comparisons between libraries, genes showing $|\log 2(\text{FoldChange})| > 1$ and a $P$ value $< 0.05$ were identified as DEGs by DEseq software 1.32.0 (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html). The expression pattern of DEGs in the ER0, ER2, ER4, ER6, ER8, ER10 and ER12 libraries were analyzed by hierarchical clustering using Pheatmap software 1.0.12 with default parameters (https://cran.r-project.org/web/packages/pheatmap/index.html). The significantly enriched GO terms examined by topGO v2.4.0, as well as significantly enriched KEGG pathways by ClusterProfiler v3.16.1 software, of all DEGs (up- and down-regulated genes) were detected with a corrected $P$ value $< 0.05$ by the hypergeometric test method (Eden et al. 2009; Mao et al. 2005).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

For qRT-PCR analysis, ten candidate DEGs (Six DEGs, TRINITY_DN11736_c0_g1, TRINITY_DN14475_c0_g1, TRINITY_DN299_c1_g1, TRINITY_DN4858_c0_g2 and TRINITY_DN5423_c0_g2 were identified as log2(FC) > 5; three DEGs, TRINITY_DN5577_c0_g1, TRINITY_DN2748_c0_g1 and TRINITY_DN5577_c0_g2, were enriched in “plant hormone signal transduction”; One DEGs, TRINITY_DN7281_c1_g1, was enriched in “tryptophan metabolism” pathway) were randomly chosen to validate the transcriptomic data. qRT-PCR was performed with the LightCycler 480 System (Roche Diagnostics, Mannheim, Germany) using PerfectStart Green qPCR Supermix (TransGen Biotech, Beijing, China). E. excelsum actin was used as the internal control and the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) was used to analyze the differential expression of candidate DEGs. Gene-specific primers were designed by Primer Premier 5.0 and are listed in Table S1. Three biological replicates and three technical replicates were performed for each candidate gene.

Results

Adventitious root formation during in vitro and ex vitro rooting

During in vitro rooting, compared with the control group, NAA did not induce ARs whereas IBA or IAA could. High concentrations of IBA and IAA inhibited rooting percentage (Fig. 1a), root number (Fig. 1c) and root length (Fig. 1e). Highest rooting percentage (72.50%) was obtained by 0.5 μM IAA at 60 d after treatment. During ex vitro rooting, NAA, IBA and IAA significantly increased rooting percentage (Fig. 1b), root number (Fig. 1d) and root length (Fig. 1f) compared with the control group. A low concentration of IBA (1 mM) most effectively induced rooting, resulting in the highest rooting percentage (72.50%) and root length (2.72 cm) at 25 d after treatment.

Ex vitro rooting induced the highest rooting percentage (98.33%) at 25 days, while highest rooting percentage during in vitro rooting was 72.50% at 60 days. Thus, ex vitro rooting induced AR from E. excelsum plantlets earlier (faster) than in vitro rooting. The samples collected from ex vitro rooting were used for the next analysis. 8 days after the 1 mM IBA ex vitro rooting treatment, AR primordia were evident, and ARs emerged from the epidermis after 10 days (Fig. 2a, b). ARs elongated, rooting percentage was almost 100% by 25 days, and plantlet survival reached 100%.

IAA and H2O2 content analysis

In the 1 mM IBA treatment during ex vitro rooting, IAA content in stem bases increased gradually from 0 to 8 days,
then dropped at 10 and 12 days. A sharp increase in IAA content was observed at 8 days (Fig. 3a). The trend of H$_2$O$_2$ content was different from that of IAA content (Fig. 3b). H$_2$O$_2$ accumulated rapidly after treatment, peaked at 2 days, then sharply decreased at 8 days. The highest content of IAA and lowest content of H$_2$O$_2$ at 8 days corresponded to the timing of AR primordia formation.

**De novo assembly and sequence analysis**

To identify genes involved in AR induction of *Euryodendron excelsum* plantlets during ex vitro rooting, 21 cDNA libraries were prepared from three repeat mRNA samples collected from 0 (ER0), 2 (ER2), 4 (ER4), 6 (ER6), 8 (ER8), 10 (ER10) and 12 (ER12) days after 1 mM IBA treatment (Table 1). The total number of raw reads produced for each library ranged from 42,845,192 to 52,575,518 with Q20 > 97.48% and Q30 > 93.42%. After filtering, the clean reads per library ranged from 39,721,158 to 49,142,018 with the percentage of clean reads > 91.07% (Table 1). Trinity software v2.5.1 was used to assemble clean reads and obtain transcripts and unigenes for subsequent analysis. The quality and length distribution of transcripts and unigenes are shown in Table 2 and Fig. S1, respectively.

The unigenes were processed in six databases to perform best hits by Blast with $E$ values < 10$^{-5}$, and inferred putative functions of the sequences were assigned. A total of 52,188 (40.15%) unigenes were matched to known genes in the NR database, 23,159 (17.82%) sequences to Pfam and 37,827 (29.10%) sequences in the Swiss-Prot database (Table 3). The NR database queries revealed that the annotated unigenes were assigned with a best score to sequences from
the top seven species (Fig. 4): *Vitis vinifera* (21.76%), *Theobroma cacao* (4.34%), *Coffea canephora* (4.01%), *Nelumbo nucifera* (3.88%), *Sesamum indicum* (3.13%), *Ziziphus jujuba* (2.67%) and *Manihot esculenta* (2.25%).

The annotation of GO terms revealed that 24,939 unigenes (19.19%) were assigned to biological processes, molecular functions, and cellular components (Fig. S2). Most annotated unigenes in biological processes were involved in “cellular process”, “metabolic process”, and “single-organism process”. In the cellular component category, most annotated unigenes were annotated as “cell”, “cell part” and “membrane”. In the molecular functions, most annotated unigenes were categorized as “binding”, “catalytic activity” and “transporter activity”.

A total of 22,160 unigenes (17.05%) and 33 pathways were assigned based on metabolism, genetic information processing, environmental information processing, cellular processes and organismal systems pathway (Fig. S3). On the basis of KEGG analysis, most unigenes were annotated into “carbohydrate metabolism” of metabolism, “translation” of genetic information processing, “signal transduction” of environmental information processing, “transport and

![Adventitious root development of *Euryodendron excelsum* shoots from 0 to 12 days after 1 mM IBA treatment during ex vitro rooting. a Phenotypic changes between 0 and 12 days after treatment. Red bars = 1 cm. b Anatomy of adventitious root development from 0 to 12 days after treatment. Red bars = 0.1 mm. Red arrows indicate the root primordium (8 days) and adventitious roots (10 days and 12 days).](image-url)
The possible functions of unigenes were predicted and classified by alignment to the eggNOG database. A total of 50,632 unigenes (38.95%) were distributed into 25 categories (Fig. S4). Among them, the NOG category “general function prediction only” represented the largest group, followed by “function unknown”, “signal transduction mechanisms”, and “posttranslational modification, protein turnover, chaperones”.

**DEGs in response to IBA-induced ex vitro rooting**

Hierarchical clustering was used to analyze the expression patterns of DEGs in ER0, ER2, ER4, ER6, ER8, ER10 and ER12 libraries with three biological replicates. These DEGs were divided into nine main clusters (Fig. S5). DEGs in cluster 1, 2, 3 and 4 always showed high expression in the ER0 library with different trends in the other five libraries. The remaining five clusters represented DEGs with high expression levels induced by IBA treatment. The highest number of up-regulated genes was observed in the ER2 library (7364) and fewest in the ER8 library (5649) (Fig. 5a). Upset plot diagram analysis showed that 4635 unigenes maintained differential expression after IBA-induced treatment from 2 to 12 days (Fig. 5b).

**GO enrichment analysis**

According to GO enrichment analysis, the degree of enrichment was measured based on the rich factor (higher rich factor represents greater enrichment), the FDR value (range from 0 to 1; a score close to 0 indicates more significant enrichment) and the number of genes enriched to a GO term. The significant enrichment GO terms of DEGs showed a few differences in the six libraries (Fig. S6). In the ER2 library, the significantly enriched terms were “monooxygenase activity”, “response to auxin”, “oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen” and “oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen”. More enrichment terms were categorized into biological processes (BP) and molecular functions (MF). In the ER4 library, more enrichment terms were categorized into cellular component (CC) and MF, and the significantly enriched terms were the same as the ER2 library, but the term “response to auxin” was replaced by “photosynthesis, light reaction”. In the ER6 and ER8 libraries, more enrichment terms were categorized into CC and MF, and “oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen” and “oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen”. More enrichment terms were categorized into biological processes (BP) and molecular functions (MF). In the ER10 library, more enrichment terms were categorized into CC and MF, and “photosynthesis, light harvesting”, “chlorophyll binding” and “photosystem I” represented the main significantly enriched terms. In the ER12 library, more enrichment terms were categorized into BP and MF, and the significantly enriched terms were “hydrogen peroxide metabolic process”, “hydrogen peroxide catabolic process” and “phenylpropanoid metabolic process”.

Besides “hydrogen peroxide metabolic process” and “hydrogen peroxide catabolic process”, several terms related to adversity stress were also identified in the GO enrichment analysis (Fig. 6). The terms “hydrogen peroxide metabolic process” and “hydrogen peroxide catabolic process” shared the same number and type of DEGs, most of which, including DEGs for the “response to oxidative stress” term, were up-regulated at 8 days after ex vitro treatment with IBA (Table S2). Most DEGs were associated with the term “response to abiotic stimulus”, followed by “response to oxidative stress” and “response to biotic stimulus”, while...
the fewest DEGs were associated with the term “response to hydrogen peroxide” (Fig. 6a). Most of the DEGs in the terms “response to abiotic stimulus” and “response to biotic stimulus” were up-regulated throughout the entire process of AR formation (Table S2). The terms “hydrogen peroxide metabolic process”, “hydrogen peroxide catabolic process” and “response to oxidative stress” encompassed 43 DEGs simultaneously (Fig. 6b), and these were mainly identified

Table 1 Statistics of output sequencing in *Euryodendron* *excelsum*

| Sample | Reads (No.) | Bases (bp) | N (%) | Q20 (%) | Q30 (%) | Clean reads (No.) | Clean reads (%) |
|--------|-------------|------------|-------|---------|---------|------------------|----------------|
| ER0_1  | 47083132    | 7062469800 | 0.002104 | 98.07   | 94.92   | 43334844         | 92.03          |
| ER0_2  | 44698062    | 6704709300 | 0.000692 | 98.24   | 95.35   | 4296590          | 92.39          |
| ER0_3  | 45235036    | 6785255400 | 0.002107 | 98.09   | 94.96   | 41952686         | 92.74          |
| ER2_1  | 44745678    | 6711851700 | 0.002111 | 98.2    | 95.26   | 41351042         | 92.41          |
| ER2_2  | 49277870    | 7391680500 | 0.002107 | 98.18   | 95.21   | 45604282         | 92.54          |
| ER2_3  | 46538306    | 6980745900 | 0.002089 | 98.16   | 95.17   | 43051642         | 92.5           |
| ER4_1  | 47269062    | 6709035300 | 0.002118 | 98.2    | 95.24   | 41605118         | 93.02          |
| ER4_2  | 44666182    | 669927300  | 0.002123 | 98.02   | 94.72   | 41816772         | 93.62          |
| ER4_3  | 51150578    | 7672586700 | 0.002111 | 98.11   | 95.02   | 46585956         | 91.07          |
| ER6_1  | 50692118    | 7603817700 | 0.002105 | 98.15   | 95.05   | 47493452         | 93.69          |
| ER6_2  | 43673982    | 6551097300 | 0.000688 | 97.48   | 93.42   | 40191318         | 92.02          |
| ER6_3  | 52517348    | 7877602200 | 0.002118 | 98.11   | 94.95   | 49142018         | 93.57          |
| ER8_1  | 49461254    | 7419188100 | 0.002101 | 98.08   | 94.96   | 45752154         | 92.5           |
| ER8_2  | 48366196    | 7254929400 | 0.000691 | 98.01   | 94.79   | 44634278         | 92.28          |
| ER8_3  | 52162670    | 782400500  | 0.00214  | 98.03   | 94.81   | 48470818         | 92.92          |
| ER10_1 | 43526700    | 652900500  | 0.001406 | 97.53   | 93.51   | 40019246         | 92.94          |
| ER10_2 | 44338864    | 600829600  | 0.001386 | 97.55   | 93.57   | 39914282         | 92.09          |
| ER10_3 | 48691108    | 7303666200 | 0.001384 | 97.52   | 93.58   | 44741570         | 91.88          |
| ER12_1 | 52575518    | 7886327700 | 0.002102 | 98.14   | 95.1    | 48722638         | 92.67          |
| ER12_2 | 42845192    | 6426778800 | 0.002116 | 98.12   | 95.08   | 39721158         | 92.7           |
| ER12_3 | 46393152    | 6950872800 | 0.002147 | 98.07   | 94.93   | 42861146         | 92.49          |

Table 2 The quality of transcripts and unigenes in *Euryodendron* *excelsum*

| Transcripts | Unigenes |
|-------------|----------|
| Total Length (bp) | 465,361,382 |
| Sequence Number | 298,338 |
| Max. Length (bp) | 17,458 |
| Mean Length (bp) | 1559.85 |
| N50 (bp) | 2401 |
| N50 Sequence No | 60,916 |
| N90 (bp) | 684 |
| N90 Sequence No | 196,779 |
| GC% | 40.07 |

| Transcripts | Unigenes |
|-------------|----------|
| Total Length (bp) | 148,606,377 |
| Sequence Number | 129,987 |
| Max. Length (bp) | 17,458 |
| Mean Length (bp) | 1143.24 |
| N50 (bp) | 1780 |
| N50 Sequence No | 23,462 |
| N90 (bp) | 467 |
| N90 Sequence No | 90,195 |
| GC% | 39.25 |

N50 (bp)—All sequences are arranged from long to short, and the sequence length are added in this order. When the added length reaches 50% of the total length of the sequence, the length of the last sequence is N50, N90 (bp)—All sequences are arranged from long to short, and the sequence length are added in this order. When the added length reaches 90% of the total length of the sequence, the length of the last sequence is N90, N50 Sequence No.—The total number of sequences that the length is greater than N50, N90 Sequence No.—The total number of sequences that the length is greater than N90, GC%—The proportion of guanidine and cytosine nucleotides among total nucleotides.

Table 3 The results of annotation of unigenes in *Euryodendron* *excelsum*

| Database | Number | Percentage (%) |
|----------|--------|----------------|
| NR       | 52,188 | 40.15          |
| GO       | 24,939 | 19.19          |
| KEGG     | 22,160 | 17.05          |
| Pfam     | 23,159 | 17.82          |
| eggNOG   | 50,632 | 38.95          |
| Swissport| 37,827 | 29.10          |
| In all databases | 8022 | 6.17 |

The fewest DEGs were associated with the term “response to hydrogen peroxide” (Fig. 6a). Most of the DEGs in the terms “response to abiotic stimulus” and “response to biotic stimulus” were up-regulated throughout the entire process of AR formation (Table S2). The terms “hydrogen peroxide metabolic process”, “hydrogen peroxide catabolic process” and “response to oxidative stress” encompassed 43 DEGs simultaneously (Fig. 6b), and these were mainly identified
KEGG enrichment analysis

KEGG pathway enrichment analysis was performed in addition to GO enrichment analysis, and the pathways significantly enriched in each stage were similar (Fig. S7). DEGs were extremely enriched in “photosynthesis–antenna proteins”, “diterpenoid biosynthesis”, “brassinosteroid biosynthesis”, “flavone and flavanol biosynthesis”, “flavonoid biosynthesis”, and “tryptophan metabolism”, et al., pathways in the ER2, ER4, ER6, ER8, ER10 and ER12 libraries. Most DEGs were enriched in “plant hormone signal transduction” and “phenylpropanoid biosynthesis” pathways.

DEGs enriched in plant hormone signal transduction pathway

KEGG pathway enrichment analysis showed that many DEGs were enriched in the “plant hormone signal transduction” pathway, and 132 up-regulated DEGs involved in auxin, cytokinin, gibberellin, abscisic acid, ethylene, brassinosteroids, jasmonic acid and salicylic acid signal transduction were identified, including genes encoding auxin-induced protein (AUX), auxin-responsive proteins (SMALL AUXIN UP RNA, SAUR; Auxin/Indole-3-Acetic Acid, AUX/IAA; Indole-3-acetic acid-amido synthetase Gretchen Hagen3, GH3), auxin transporter-like protein (AUX1/LAX), ethylene-responsive transcription factor (ERF), and others (Table S3). The up-regulated expression of 24 DEGs was maintained after the IBA-induced ex vitro treatment, mainly SAUR50, SAUR32, SAUR36, IAA11, IAA13, IAA16, GH3.1, GH3.3, GH3.5, GH3.6, and ERF.

A total of 42 up-regulated genes related to auxin-responsive proteins, including 13 IAA, 19 SAUR and 10 GH3 genes, were identified in the significantly enriched pathway “plant hormone signal transduction” (Fig. 7). Most of those genes were differentially expressed in early response to IBA treatment, at 2 and 4 days. Four IAA and three SAUR genes sustained up-regulated expression after IBA-induced treatment. IAA1 (TRINITY_DN5977_c2_g1) showed high expression with log2(FC) > 4 at ER2, ER4, ER6, ER8, and ER10 stages, and even with log2(FC) > 8 at the ER2 stage. IAA30 (TRINITY_DN1790_c2_g1) showed high expression with log2(FC) > 4 at all stages, and even with log2(FC) > 8 at the ER2 and ER4 stages. Some DEGs were specifically differentially expressed at certain stages, such as TRINITY_DN14445_c0_g2 at ER2, TRINITY_DN58019_c0_g2 at ER10, and TRINITY_DN1958_c1_g2, TRINITY_DN25899_c0_g1, TRINITY_DN58019_c0_g1 and TRINITY_DN9289_c0_g1 at ER12. Five GH3 genes were up-regulated at all stages, demonstrating log2(FC) > 4 for TRINITY_DN2748_c0_g1 and TRINITY_DN2800_c0_g1, and log2(FC) > 8 for TRINITY_DN5152_c0_g1.

The 12 up-regulated genes related to auxin-induced proteins, identified as AX6B, AX10A, A10A5, AX15A, AX22, AUX22D, and AUX28, were sharply enriched in the “plant hormone signal transduction” pathway (Fig. 8a). Most of those genes were also differentially expressed at an early stage (2, 4 days) after IBA treatment. Among these genes, TRINITY_DN39834_c0_g1 and TRINITY_DN31248_c0_g1 were extremely highly differentially expressed with log2(FC) > 8 in the ER0 vs ER2 and ER0
vs ER4 comparisons. Four DEGs were up-regulated with log2(FC) > 1 by IBA treatment under ex vitro rooting in the ER0 vs ER2, ER0 vs ER4, ER0 vs ER6 and ER0 vs ER8 comparisons while TRINITY_DN5677_c0_g1 maintained up-regulated expression at all stages.

In addition, five up-regulated LAX genes (Fig. 8b) were significantly enriched in the “plant hormone signal transduction” pathway. Only TRINITY_DN9654_c0_g1 was up-regulated at all stages while the other four LAX genes were up-regulated at ER10 and ER12, except for TRINITY_DN380_c4_g1, which was up-regulated at ER6.

QRT-PCR analysis of gene expression

To further validate the results from the RNA-seq data, 10 candidate DEGs related to adventitious root formation were selected for qRT-PCR analysis of E. excelsum samples that were collected 0, 2, 4, 6, 8, 10 and 12 days after 1 mM IBA treatment during ex vitro rooting. In the seven time points, the expression trend of the unigenes from qRT-PCR and RNA-seq analysis were largely consistent (Fig. 9). These results demonstrate that the transcriptome data accurately reflects the ex vitro response of IBA-induced AR formation of E. excelsum plantlets.
Discussion

AR development is a vital step in plant vegetative propagation, such as in vitro propagation and cuttings. Rooting recalcitrance is a critical factor limiting the application and further development of vegetative propagation (Diaz-Sala 2020; Stevens et al. 2018). IBA is the most frequently used plant hormone for clonal propagation in horticulture and forestry. Although IAA is a primarily native auxin in plants, IBA is more stable and effective in promoting ARs (Ludwig- Müller et al. 2005; Quan et al. 2017; Rout 2006). It is necessary to screen PGRs to find the optimal species-concentration ratio for AR induction during in vitro culture. In this study, IBA and IAA treatment significantly promote AR formation of *E. excelsum*, especially during ex vitro rooting. Furthermore, ex vitro rooting was more suitable for *E. excelsum* plantlets, with a higher rooting percentage and earlier rooting than in vitro rooting. Ex vitro rooting of shoots has also been applied to many difficult-to-root woody plant species, such as pistachio (Benmahioul et al. 2012), *Dalbergia sissoo* Roxb. (Vibha et al. 2014) and *Bauhinia racemosa* Lam (Sharma et al. 2017). In general, the chances of root damage during transplantation to substrates are less during ex vitro rooting, and plantlets tend to be more vigorous, allowing them to cope with environmental stresses during hardening (Arya et al. 2003; Vengadesan and Pijut 2009). Thus, ex vitro rooting is an obvious choice for AR induction during the micropropagation of woody species with further improvement in the choice of PGRs, substrates and other factors.

*E. excelsum* plantlets experienced a radical environmental change from in vitro aseptic conditions to open ex vitro rooting conditions, which may constitute an adversity stress. In the annotation and enrichment analysis of GO terms, we identified multiple DEGs involved in H$_2$O$_2$-related biological activities, oxidative stress, abiotic and biotic stimulus. AR formation is also a stress response of plants under adversity stress, and plays a key function in the adaptation of plants to abiotic and biotic stresses (Bellini et al. 2014; Steffens and Rasmussen 2016). The external environmental change may stimulate oxidative damage and increase the production of reactive oxygen species in plants (You and Chan 2015). H$_2$O$_2$ is viewed mainly as a type of reactive oxygen species and a signaling messenger of many biological processes in plants, such as fruit growth and development (Khandaker et al. 2012), leaf senescence (Lin et al. 2019), stomatal closure (Zhang et al. 2019a), and root growth (Xiong et al. 2019). H$_2$O$_2$ and IBA may also act synergistically to regulate adventitious rooting, dependent on the auxin pathway, in marigold explants (Liao et al. 2011). The exogenous application of H$_2$O$_2$ to cucumber plants significantly increased the emergence of ARs (Li et al. 2016b). In this study, the wide fluctuation of endogenous IAA and H$_2$O$_2$ content in *E. excelsum* plantlets were observed at the stage of root primordia formation. And most DEGs involved in significantly enriched pathway of “plant hormone signal transduction” were up-regulated at the stage corresponded to the timing of H$_2$O$_2$ accumulation. Those results indicate that adversity stress may have a positive effect on AR induction of *E. excelsum* plantlets under the synergistic action of

![Graph and Venn diagram](image-url)
H$_2$O$_2$ and PGRs. The synergistic action of H$_2$O$_2$ and PGRs on AR induction from *E. excelsum* plantlets will be revealed by additional research.

AR formation involves a series of responses by genes, proteins and metabolites. Multiple biological activities and pathways have specific roles during AR development (de Almeida et al. 2020; Wei et al. 2014). In mungbean seedlings, KEGG pathway enrichment during transcriptomic analysis showed that ribosome biogenesis, plant hormone signal transduction, pentose and glucuronate interconversions, photosynthesis, phenylpropanoid biosynthesis, sesquiterpenoid and flavonoid biosynthesis, and phenylalanine metabolism were the pathways most highly regulated by IBA-induced AR formation, indicating their potential contribution to adventitious rooting (Li et al. 2016a). For apple rootstocks, the most heavily enriched KEGG pathways involved in AR formation were metabolic, biosynthesis of secondary metabolites, plant hormone signal transduction, phenylpropanoid biosynthesis and phenylalanine metabolism pathways, etc. (Li et al. 2018). In sugarcane shoots, DEGs associated with plant hormone signaling, flavonoid and phenylpropanoid biosynthesis, cell cycle, and cell wall modification, and transcription factors were involved in AR formation (Li et al. 2020). During AR development of *E. excelsum*, we found that more DEGs were enriched in “plant hormone signal transduction” and “phenylpropanoid
biosynthesis” pathways, similar to a number of previous studies. Therefore, we conclude that these two pathways have a vital influence on AR formation in *E. excelsum* plants.

IAA is the most abundant natural auxin, and endogenous IAA is closely related to the development of ARs in plants. The conversion of exogenous hormones to endogenous auxin and the synthesis of auxin are key factors regulating AR development (Olatunji et al. 2017). Tissue that produces ARs requires high levels of auxin, and the enrichment of high concentrations of auxin depends on polar auxin transport (Ahkami et al. 2013; Garrido et al. 2002). Thus, genes related to the synthesis, signaling and polar transport of auxin, like *AUX*, *LAX*, and *PIN*, are closely related to plant adventitious rooting (Druege et al. 2016). For example, auxin influx carriers *MiAUX3* and *MiAUX4* might play important roles during AR formation in mango cotyledon segments, and the expression levels of *MiAUX3* and *MiAUX4* resulted in a significant promotive effect of IBA on adventitious rooting (Li et al. 2012). Papaya plantlets not exposed to IBA could not form ARs and displayed a low expression of all auxin transporter genes in stem base tissues whereas IBA-treated plants were able to produce ARs and showed significantly increased expression of most auxin transporter genes, especially *CpLAX3* and *CpPIN2* (Estrella-Maldonado et al. 2016). In *E. excelsum*, DEGs for *AUX* and *LAX*, which were significantly enriched in plant hormone signal transduction, showed a high fold change during AR development. This implies that the expression patterns of those genes were linked to AR induction from *E. excelsum* plantlets.

AUX/IAA protein is an early auxin response protein that always participates in the auxin signaling pathway by interacting with auxin response factor (ARF) protein or other genes (Salehin et al. 2015). During AR formation in petunia cuttings, the expression of genes of the Aux/IAA family showed strong temporal variation, supporting their important role in the induction and transition to subsequent root formation phases (Druege et al. 2014). The auxin receptor (TRANSPORT INHIBITOR1) TIR1 homolog gene, *PagFBL1*, interacted strongly with both *PagIAA28.1* and *PagIAA28.2* in the presence of NAA to regulate AR induction in poplar stem segments (Shu et al. 2019). In *Arabidopsis thaliana*, Aux/IAA proteins, IAA6, IAA9, and IAA17, interacted with ARF6 and/or ARF8 and likely repressed their activity in AR development, and complexed with TIR1 and (AUXIN-SIGNALLING F-BOX) AFB2 to form specific sensing to modulate jasmonic acid homeostasis and control AR initiation (Lakehal et al. 2019). In this study, 13 IAA genes were significantly enriched in the plant hormone signal transduction pathway, suggesting a significant relationship between AUX/IAA and AR formation in *E. excelsum*. The mechanism and interaction with other IAA genes would need to be revealed in future research.

SAUR, the largest family of early auxin response genes in plants, mediate the regulation of several aspects of plant growth and development (Ren and Gray 2015). SAUR proteins showed positive or negative effects on primary, lateral and adventitious root development. In *A. thaliana*, plants overexpressing *SAUR41* exhibited increased...
primary root growth and a higher number of lateral roots (Kong et al. 2013). AtSAUR15 acts downstream of the auxin response factors ARF6,8 and ARF7,19 to regulate auxin signaling-mediated lateral root and AR formation, and plants overexpressing AtSAUR15 exhibit more lateral roots and ARs (Yin et al. 2020). In contrast to AtSAUR41 and AtSAUR15, overexpression of OsSAUR39 in rice resulted in reduced root elongation and lateral root development (Kant et al. 2009). SAUR proteins may display a species- or type-dependent positive function in AR formation. In E. excelsum, three SAUR genes maintained up-regulated expression after IBA-induced treatment, indicating a close association with AR formation.

We also found several highly up-regulated GH3 genes at all stages of AR formation in E. excelsum. GH3 proteins are also an early auxin response protein, play a crucial role in conjugating IAA to amino acids, and are critical in maintaining auxin homeostasis (Brunoni et al. 2020). Three GH3 genes, GH3.3, GH3.5, and GH3.6, were required for fine-tuning AR initiation in A. thaliana hypocotyls (Gutierrez et al. 2012). In cucumber hypocotyls, salicylic acid plays an inducible role in AR formation through competitive inhibition of the auxin conjugation enzyme CsGH3.5, and salicylic acid-induced IAA accumulation was also associated with the enhanced expression of CsGH3.5 (Dong et al. 2020). In apple plants, overexpression of MsGH3.5 significantly reduced the content of free IAA and increased the content of some IAA-amino acid conjugates, and MsGH3.5-overexpressing lines produced fewer ARs than the control (Zhao et al. 2020). Those results demonstrated that GH3 proteins were intricately involved in AR development, but did not only perform a positive role.

**Conclusion**

Here, we confirmed that ex vitro rooting was an obvious choice for AR formation during the micropropagation of E. excelsum plantlets. DEGs enriched in the pathway of plant hormone signal transduction played a crucial role in AR formation. H2O2 produced by environmental stimulation might be related to AR induction in E. excelsum ex vitro by the synergistic action with IBA, ultimately regulating the level of endogenous IAA. The knowledge gained from this study will help researchers understand the molecular traits of IBA-based regulation of adventitious rooting of E. excelsum plantlets. These results will provide technical support for the ecological protection of this rare and endangered species and are important for research and commercial applications aimed at overcoming rooting recalcitrance in plant species of economic value, in difficult-to-root woody plants, or in rare or endangered plants.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02226-9.

**Acknowledgements** We thank Personal Biotechnology Co., Ltd. for their skilful support of RNA-sequencing.

**Author contributions** XHZ, YL, KLW, FL, JATdS, GHM and SJZ designed the experiment and provided guidance on the study. YPX and SYC prepared samples for AR induction and RNA-seq analysis. XHC, TZ, BYG and MYN performed the statistical analysis on the determination of IAA and H2O2 content, and RNA-seq data. ZPW, YYY, XCY and JHP participated in the experiment of AR induction and anatomical analysis. YPX, JATdS and SYC were involved in statistical analyses and co-wrote the manuscript. All authors wrote, read and approved the manuscript.

**Funding** This work was supported by the National Natural Science Foundation of China Youth Fund (32100311), National Key Research Plan of China (Grant No.: 2016YFC0503104) and Guangdong Science and Technology Program (Number: 2015B020231008).

**Data availability** All data generated or analyzed during this study are included in this published article and its supplementary information files. The RNA-seq data has been deposited in the Sequence Read Archives Database (https://www.ncbi.nlm.nih.gov/sra/) under accession number PRJNA723111 (http://www.ncbi.nlm.nih.gov/bioproject/723111).

**Declarations**

**Conflict of interest** The authors declare that they have no competing interests.

**Ethical approval** Specific permission was not required for plant collection at the mentioned locations.

**Consent for publication** Not applicable.

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Authors and Affiliations

Yuping Xiong1,2 · Shuangyan Chen3,4 · Zhenpeng Wei5 · Xiaohong Chen1,2 · Beiyi Guo1,2 · Ting Zhang1,2 · Yuying Yin1,2 · Xincheng Yu5 · Meiyun Niu1,2 · Xinhua Zhang1 · Yuan Li1 · Kunlin Wu1 · Lin Fang1 · Jaime A. Teixeira da Silva6 · Guohua Ma1 · Songjun Zeng1

Yuping Xiong
13838553436@163.com
Shuangyan Chen
2831716403@qq.com
Zhenpeng Wei
1076819258@qq.com
Xiaohong Chen
512740674@qq.com
Beiyi Guo
294471191@qq.com
Ting Zhang
1790994058@qq.com
Yuying Yin
15617188299@163.com
Xincheng Yu
604675055@qq.com
Jinhui Pang
191901552@qq.com
Meiyun Niu
497362954@qq.com
Xinhua Zhang
xhzhang@scib.ac.cn
Yuan Li
24459755@qq.com
Kunlin Wu
wkl8@scib.ac.cn
Lin Fang
fllm1@hotmail.com
Jaime A. Teixeira da Silva
jaimetex@yahoo.com

1 Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou 510650, China
2 University of Chinese Academy of Sciences, Beijing 100039, China
3 Institute of Nanfan & Seed Industry, Guangdong Academy of Sciences, Guangdong 510316, China
4 Zhanjiang Sugarcane Research Center, Guangzhou Sugarcane Industry Research Institute, Zhanjiang 524300, Guangdong, China
5 Zhongkai University of Agriculture and Engineering, Guangzhou 510650, China
6 Independent researcher, P.O. Box 7, Miki-cho, Ikenobe 3011-2, Kagawa-ken, Takamatsu 761-0799, Japan