Transcriptomic Analyses Provide Insight Into Adventitious Root Formation of *Euryodendron Excelsum* H. T. Chang During *Ex Vitro* Rooting

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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 d. During *ex vitro* rooting, there was a wide fluctuation of endogenous levels of indole-3-acetic acid (IAA) and hydrogen peroxide (H_2O_2) at the stage of root primordia formation. Transcriptome analysis revealed multiple differentially expressed genes (DEGs) involved in AR development. DEGs involved in plant hormone signal transduction, such as genes encoding auxin-induced protein, auxin-responsive protein, and IAA-amido synthetase Gretchen Hagen3, and in response to H_2O_2, 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. H_2O_2, 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 H_2O_2 produced by environmental stimulation and IBA played crucial role in the regulation of AR formation from *E. excelsum* plantlets during micropropagation.

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 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 mg/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 mg/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 (Barpete et al 2014, Guo et al 2019, Nourissier and Monteuuis 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 (Benmahioul 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 euramericana* (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 GB 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 significantly different from controls 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 d 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 (Mackin), 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 (Mackin). 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 (H_2O_2) content

To analyze IAA and H_2O_2 content, the same method and growth conditions were employed as for histological analysis. Material was stored at -80℃. Three biological replicates of 10 cut stem bases were harvested as 0.1 g fresh weight (FW) to assess endogenous IAA and H_2O_2 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 from controls at $P < 0.05$.

Isolation of RNA and cDNA library construction

The same samples used to analyze IAA and H_2O_2 content were employed for RNA-seq analysis. Samples collected from 0, 2, 4, 6, 8, 10, and 12 d were marked as ER0, ER2, ER4, ER6, ER8, ER10, ER12, respectively, and stored at -80℃. The Column Plant RNA_{OUT} Extraction kit (Tiandz, 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

Empty reads, adapter sequences, and low-quality reads were discarded form raw reads to obtain clean reads. The *de novo* assembly of high-quality reads was performed by Trinity software (version 2.5.1, https://github.com/trinityrnaseq/trinityrnaseq/wiki). 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://eggnog.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 |log2FoldChange| > 1 and a *P* value < 0.05 were identified as DEGs by DEseq software (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 (https://cran.r-project.org/web/packages/pheatmap/index.html). The significantly enriched GO terms and KEGG pathways of DEGs 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

Ten candidate DEGs in the significant enrichment KEGG pathway were randomly chosen for qRT-PCR analysis 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 $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used to analyze the differential expression of candidate DEGs. Gene-specific primers 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. 1b)

and root length (Fig. 1c). Highest rooting percentage (72.50%) was obtained by 0.5 μM IAA 60 d after treatment. During *ex vitro* rooting, NAA, IBA and IAA significantly increased rooting percentage (Fig. 1d), root number (Fig. 1e) 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 (98.33%) and root length (2.72 cm) 25 d after treatment.

Ex vitro rooting induced the highest rooting percentage (98.33%) at 25 d, while highest rooting percentage during *in vitro* rooting was 72.50% at 60 d. 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. Eight days after the 1 mM IBA *ex vitro* rooting treatment, AR primordia were evident, and ARs emerged from the epidermis after 10 d (Fig. 2). ARs elongated, rooting percentage was almost 100% by 25 d, and plantlet survival reached 100%.

IAA and H_2O_2 content analysis

In the 1 mM IBA treatment during *ex vitro* rooting, IAA content in stem bases increased gradually from 0 to 8 d, then dropped at 10 and 12 d. A sharp increase in IAA content was observed at 8 d (Fig. 3a). The trend of H_2O_2 content was different from that of IAA content (Fig. 3b). H_2O_2 accumulated rapidly after treatment, peaked at 2 d, then sharply decreased at 8 d. The highest content of IAA and lowest content of H_2O_2 at 8 d corresponded to the timing of AR primordia formation.

De novo assembly and sequence analysis

To identify genes involved in AR induction of *E. 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 (ER) and 12 (ER12) d 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%. The sequenced raw data was submitted to the SRA at the NCBI database with the following accession numbers: SRR14278060, SRR14278059, SRR14278048, SRR14278046, SRR14278045, SRR14278044, SRR14278043, SRR14278042, SRR14278041, SRR14278040, SRR14278058, SRR14278057, SRR14278056, SRR14278055, SRR14278054, SRR14278053, SRR14278052, SRR14278051, SRR14278050, SRR14278049, and SRR14278047. 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 was used to assemble clean reads and obtain transcripts and unigenes for subsequent analysis. The quality of transcripts and length distribution of unigenes are shown in Fig. S1.

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 2). 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 catabolism” of cellular processes, and “endocrine system” of organismal system.

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 clusters 1, 2, 3 and 4 always showed high expression in the ER0 library with different trends in the other six 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 (7,364) and fewest in the ER8 library (5,649) (Fig. 5a). Upset plot diagram analysis showed that 4,635 unigenes maintained differential expression after IBA-induced treatment from 2 to 12 d (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” and “oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen”. In the ER4 library, 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, “monooxygenase activity” and “photosystem” were listed as significantly enriched terms. In the ER10 library, “photosynthesis, light harvesting”, “chlorophyll binding” and “photosystem I” represented the main significantly enriched terms. In the ER12 library, 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 d 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 as genes related to cationic peroxidase and peroxidase (Table S2).

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” and “flavonoid biosynthesis” 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, cytokinine, 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*, *GH3.5*, *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

d. Four *IAA* and three *SAUR* genes sustained up-regulated expression after IBA-induced treatment. *IAA11* (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*, *AUX22*, *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 d) 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 were selected for qRT-PCR analysis of *E. excelsum* samples that were collected 0, 2, 4, 6, 8, 10 and 12 d 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-Muller 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 abiotic stress. In the annotation and enrichment analysis of GO terms, we identified multiple DEGs involved in H_2O_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 may stimulate oxidative damage and increase the production of reactive oxygen species in plants (You and Chan 2015). H_2O_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 2015). H_2O_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_2O_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_2O_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_2O_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 H_2O_2 and PGRs. The synergistic action of H_2O_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. H_2O_2 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 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.

Abbreviations

AR	Adventitious root
AUX	Auxin-induced protein
AUX/IAA	Auxin/Indole-3-Acetic Acid
AUX/LAX	Auxin transporter-like protein
ARF	Auxin response factor
BA	6-Benzyladenine
cDNA	Complementary deoxyribonucleic acid
DEG	Differentially expressed gene
eggNOG	Evolutionary genealogy of genes: Non-supervised Orthologous Groups
ERF	Ethylene-responsive transcription factor
FC	Fold change
FPKM	Fragments per kilobases per million mapped reads
FW	Fesh weight
GH3	Indole-3-acetic acid-amido synthetase Gretchen Hagen3
GO	Gene ontology
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
NAA	α-Naphthaleneacetic acid
NCBI	National Center for Biotechnology Information
NR	NCBI non-redundant protein sequences
PCR	Polymerase chain reaction
QRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RNA	Ribonucleic acid
RNA-seq	High-throughput RNA-sequencing
SA	Salicylic acid
SAUR	SMALL AUXIN UP RNA
WPM	Woody plant medium

Declarations

Ethics approval and consent to participate

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

Consent for publication

Not applicable.

Data and Materials 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).

Conflicts of interest

The authors declare that they have no competing interests.

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Authors' 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 H_2O_2 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.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

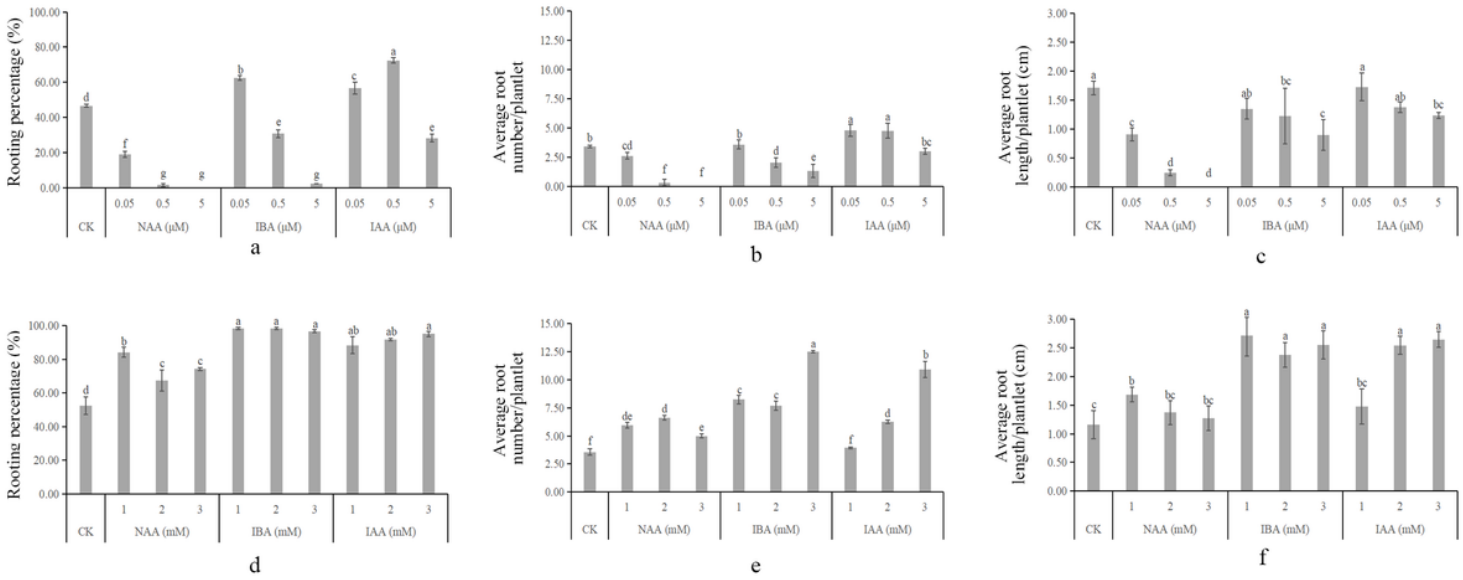

Figure 1

Adventitious root induction of Euryodendron excelsum shoots during in vitro rooting at 60 d (a, rooting percentage; b, root number; c, root length) and ex vitro rooting at 25 d (d, rooting percentage; e, root number; f, root length) after treatment. Bars indicate means±SE. Different letters indicate statistically significant differences compared with the control (CK) based on Duncan's multiple range test ($P < 0.05$) for the designated treatments. Forty shoots were prepared for each treatment, and three replicates were performed for each treatment.

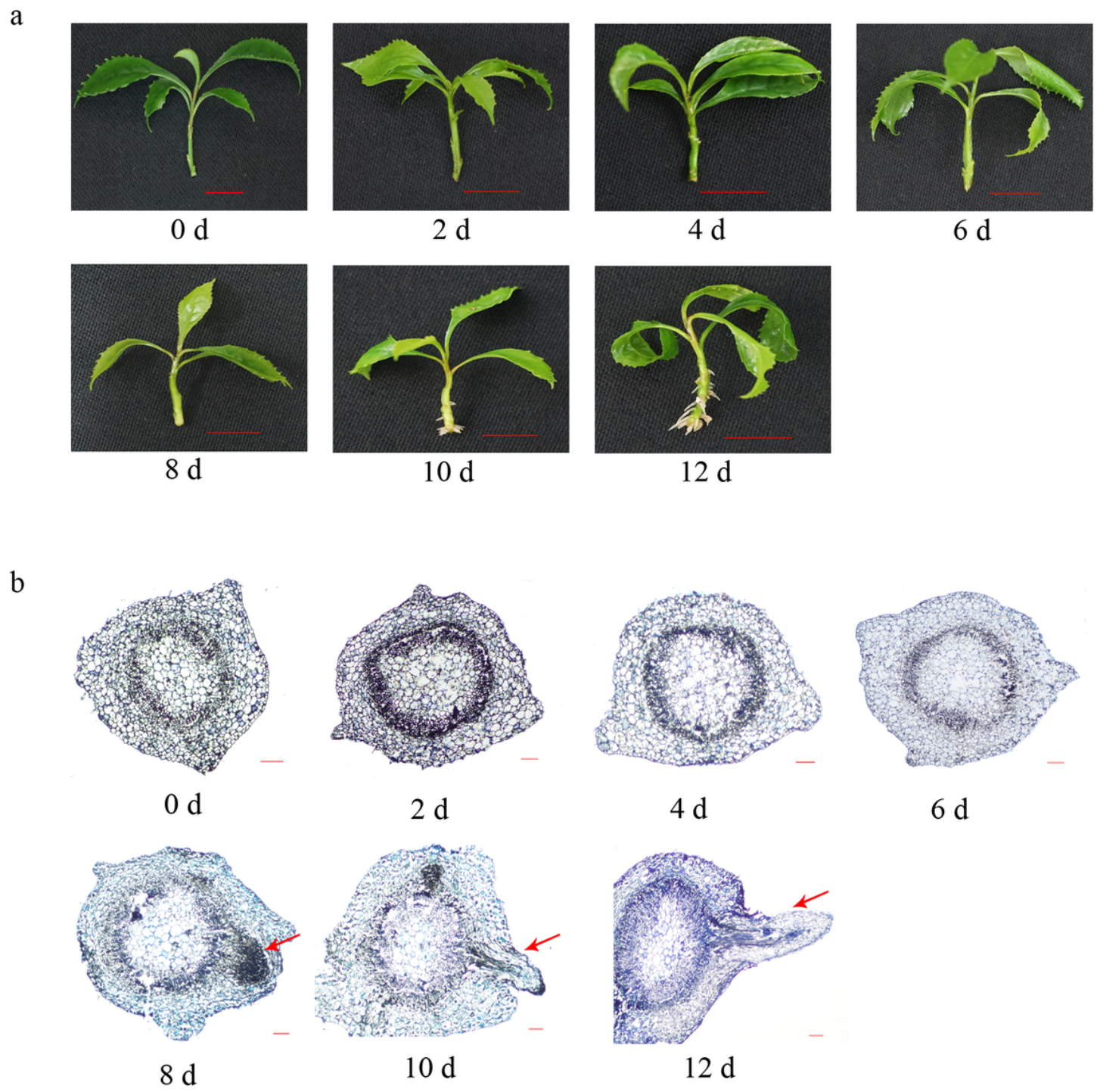

Figure 2

Adventitious root development of Euryodendron excelsum shoots from 0 to 12 d after 1 mM IBA treatment during ex vitro rooting. (a) Phenotypic changes between 0 and 12 d after treatment. Red bars = 1 cm. (b) Anatomy of adventitious root development from 0 to 12 d after treatment. Red bars = 0.1 mm. Red arrows indicate the root primordium (8 d) and adventitious roots (10 d and 12 d).

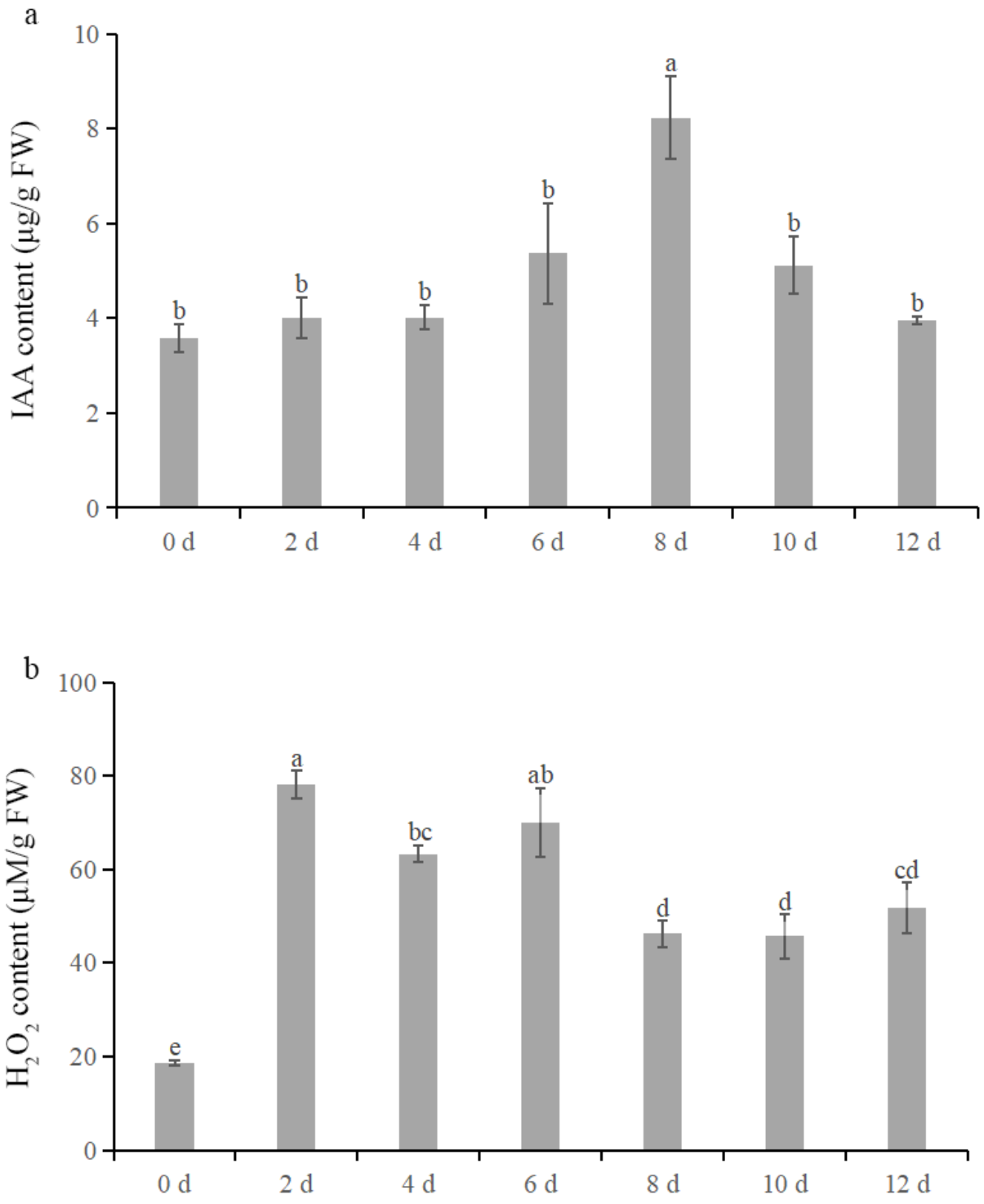

Figure 3

Determination of endogenous IAA (a) and H2O2 (b) content in stem bases of Euryodendron excelsum. Bars indicate means±SE. Different letters indicate statistically significant differences compared with the control (0 d) based on Duncan's multiple range test ($P < 0.05$) for the designated treatments. Three biological replicates of 10 cut stem bases were harvested as 0.1 g fresh weight (FW) to assess endogenous IAA and H2O2 content.

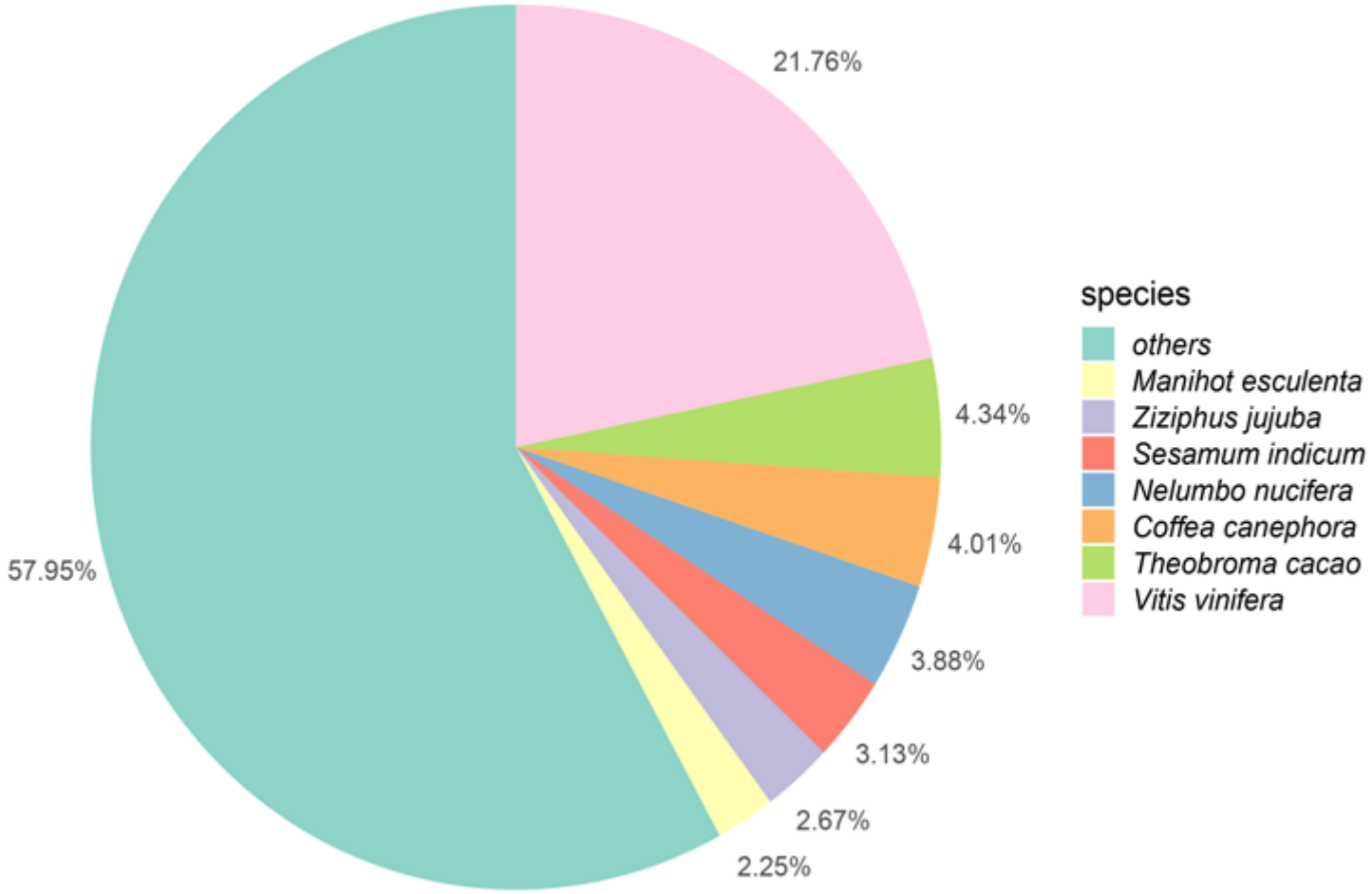

Figure 4

Species distribution of the top BLAST hits against the NR database in Euryodendron excelsum. Pie charts were generated by R software.

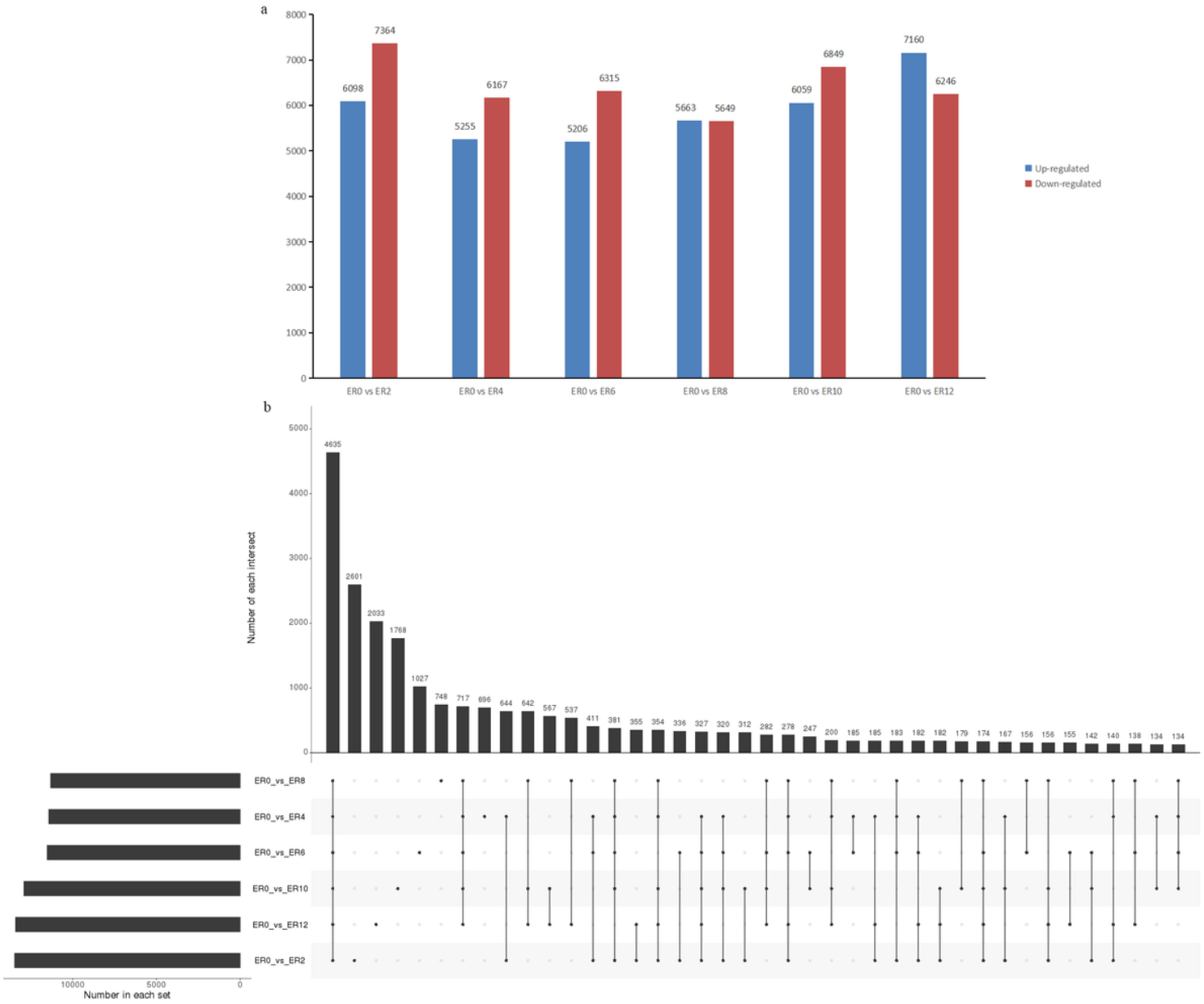

Figure 5

Number of DEGs and Upset plot diagram analysis of DEGs during adventitious root development of Euryodendron excelsum. Upset plot diagram was generated by R software. (a) Number of up- and down-regulated DEGs in 2 (ER2), 4 (ER4), 6 (ER6), 8 (ER8), 10 (ER10) and 12 (ER12) d treatments compared with 0 d (ER0). The x-axis represents the time point after IBA treatment during ex vitro rooting, while the y-axis represents the number of DEGs; (b) Upset plot diagram analysis of the DEGs. The connections on the x-axis between points in vertical lines represent the intersection between corresponding data sets, while the y-axis represents the number of DEGs in each intersection.

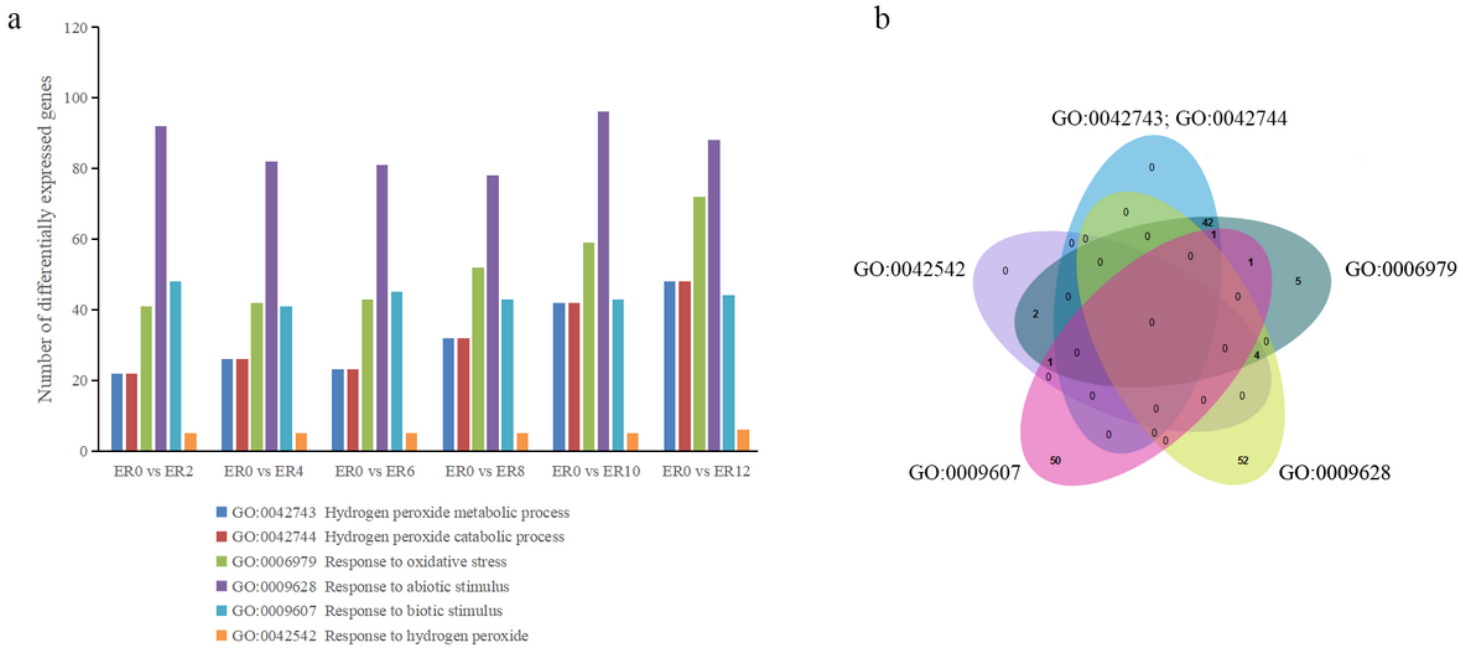

Figure 6

DEGs related to adversity stress in GO enrichment analysis. (a) Number and categories of DEGs. (b) Venn diagram analysis of up-regulated DEGs related to adversity stress.

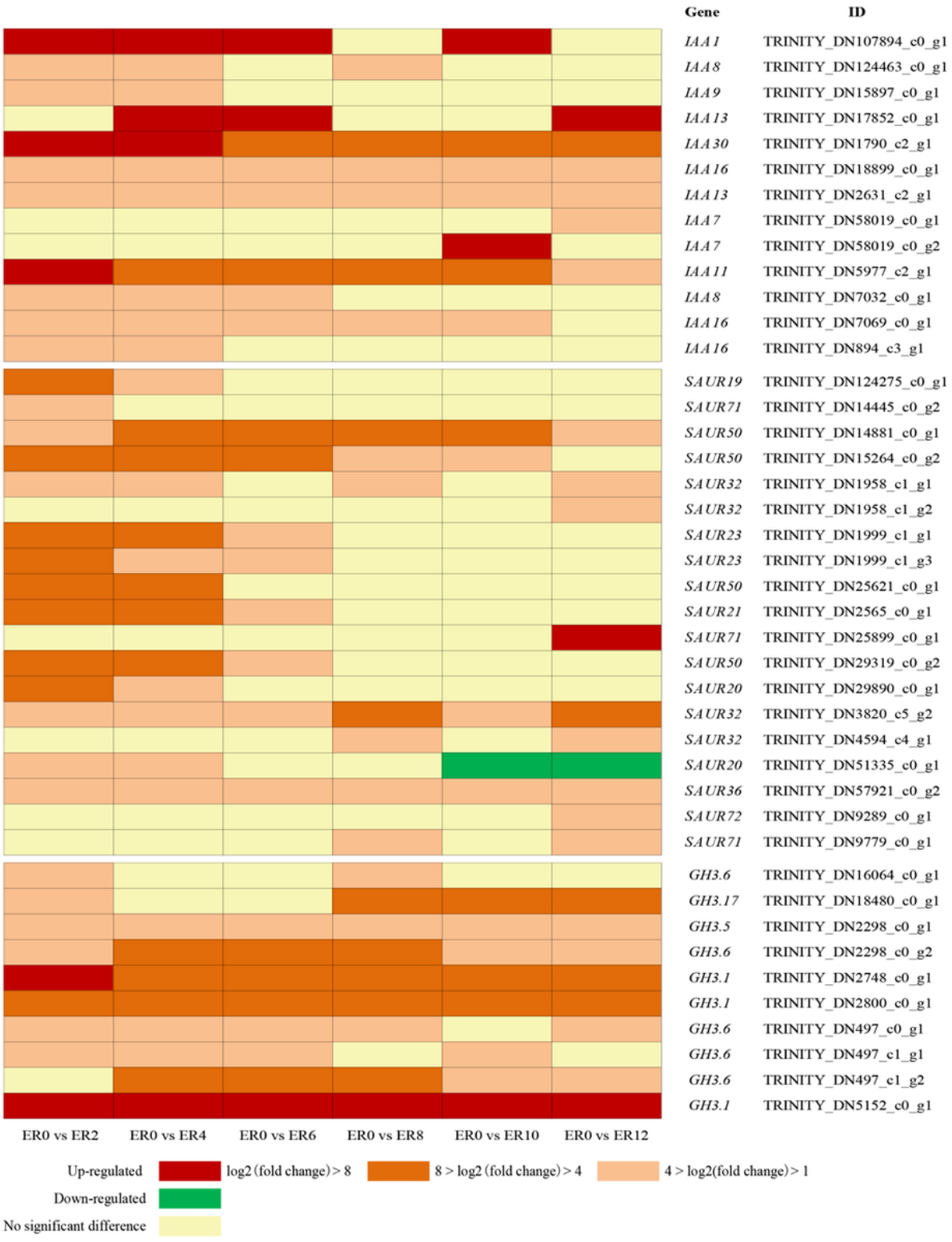

Figure 7

Analysis of the fold changes of up-regulated genes related to AUX/IAA, SAUR and GH3 in Euryodendron excelsum determined by RNA-seq. Graphs were generated by Microsoft Excel and Adobe Photoshop CC 2018.

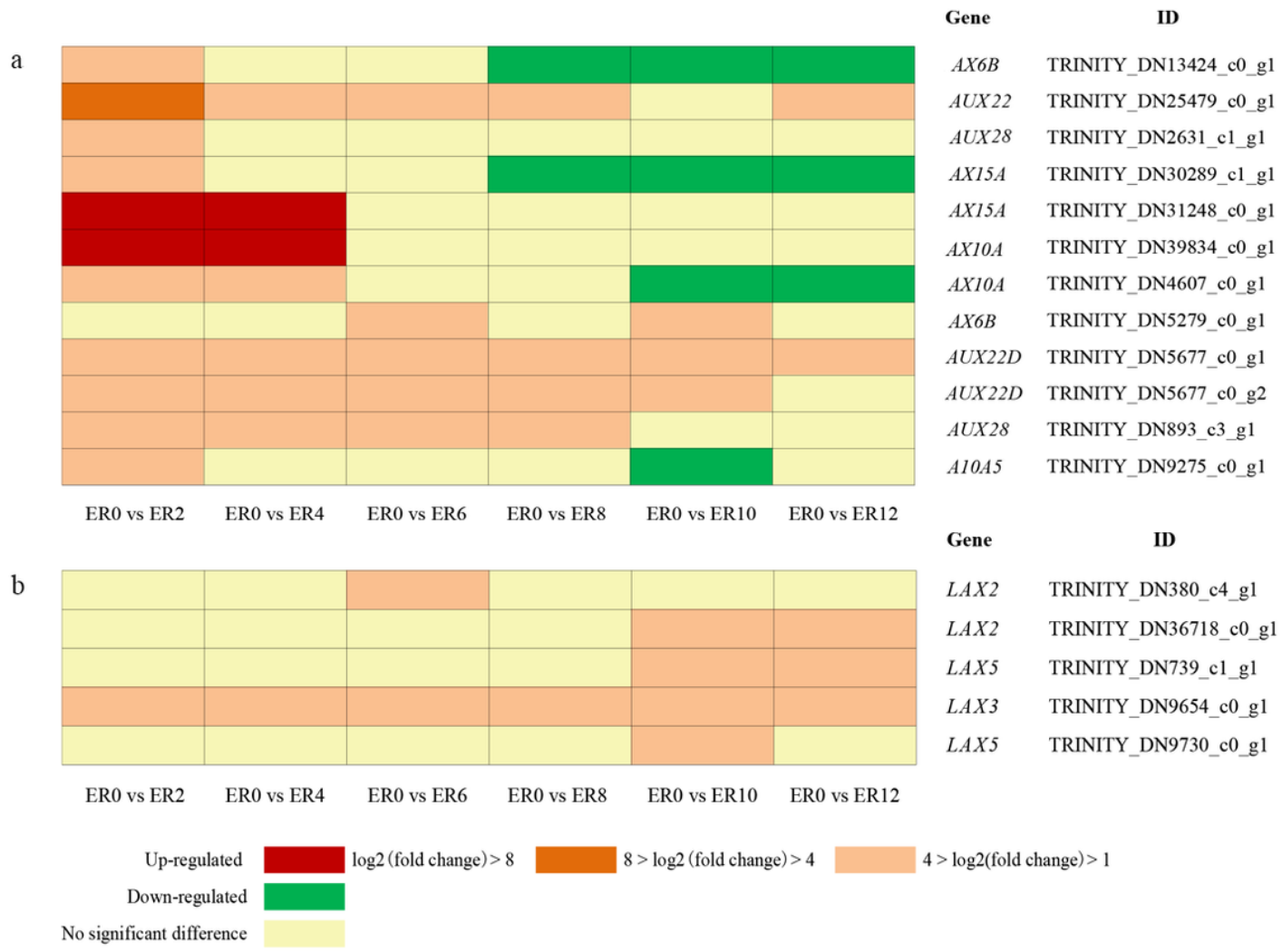

Figure 8

Analysis of the fold changes of up-regulated genes related to AUX (a) and AUX1/LAX (b) during adventitious root development of Euryodendron excelsum determined by RNA-seq. Graphs were generated by Microsoft Excel and Adobe Photoshop CC 2018.

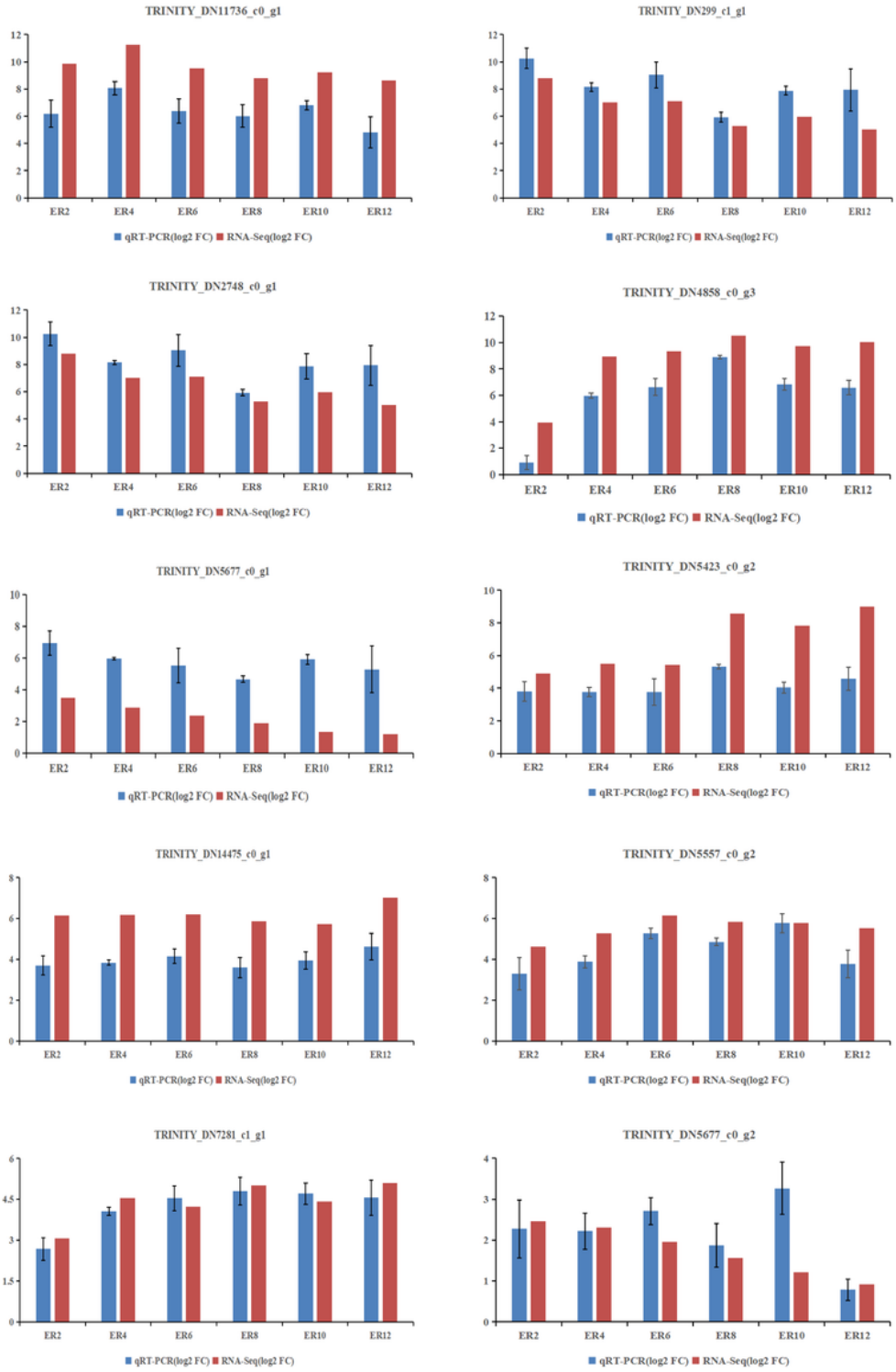

Figure 9

Analysis of the fold changes of 10 candidate genes in Euryodendron excelsum determined by RNA-seq and qRT-PCR. Bars indicate means±SD. The x-axis represents the time point after each treatment while the y-axis represents the log2(fold change). Graphs were generated by Microsoft Excel and Adobe Photoshop CC 2018.

Supplementary Files

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