



## Research article

# Transcriptome differences between 20- and 3,000-year-old *Platycladus orientalis* reveal that ROS are involved in senescence regulation



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## ABSTRACT

**Background:** *Platycladus orientalis* has an extremely long life span of several thousands of years, attracting great interests in the mechanisms involved in such successful senescence regulation and resistance at physiological and molecular levels.

**Results:** The levels of reactive oxygen species (ROS) were higher in 3,000-year-old than in 20-year-old *P. orientalis*, and the activities of GR and GSH demonstrated the same trend. We produced and analyzed massive sequence information from pooled samples of *P. orientalis* through transcriptome sequencing, which generated 51,664 unigenes with an average length of 475 bp. We then used RNA-seq analysis to obtain a high-resolution age-course profile of gene expression in 20- and 3,000-year-old *P. orientalis* individuals. Totally, 106 differentially expressed genes were obtained, of which 47 genes were downregulated and 59 upregulated in the old tree. These genes were involved in transcription factors, hormone-related responses, ROS scavengers, senescence-related responses, stress response, and defense and possibly play crucial roles in tackling various stresses in the 3,000-year-old *P. orientalis* during its life time. The expression patterns of genes related to ROS homeostasis further indicated that the high ability of ROS scavenging could be helpful for the 3,000-year-old *P. orientalis* to resist senescence.

**Conclusions:** This study provides a foundation for the elucidation of senescence resistance through molecular studies and the discovery of useful genes in *P. orientalis*.

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## 1. Introduction

Trees can live several hundreds or, even, many thousands of years. Living trees of extreme age include a 4,000-year-old *Pinus longaeva* in California, USA [1], and a more than 3,000-year-old *Platycladus orientalis* in the Yellow Emperor's Mausoleum of Shaanxi, China [2]. Normally, tree growth tends to slow, or even stop, with increasing age and size [3]. However, recent studies showed no significant age-related differences between a 4,713- and a 23-year-old bristlecone pine [4,5]. Increased telomere length and telomerase activity may both directly or indirectly contribute to the increased lifespan of bristlecone pine [6]. Why do such trees live so long? A lack of information about plant longevity has hindered attempts to assess the mechanisms involved and identify related factors [7,8]. This has attracted much attention

from plant researchers interested in senescence resistance [3]. To date, information on senescence has mainly come from model plants, and much less is known about senescence in perennials [9,10].

Senescence may be considered a strategy of plants that allows them to adapt to prevailing environmental conditions [11]. During senescence, increased amounts of reactive oxygen species (ROS) are produced, along with an increase in proteolytic activity. These excess ROS damage proteins, nucleic acids, and membrane lipids in plant cells [12]. ROS homeostasis is maintained by a group of molecules with antioxidant activity [13], including well-known anti-oxidative enzymes such as catalase (CAT), peroxidases (PODs), and superoxide dismutase (SOD) [14].

Studies on physiology and genetics show that the senescence process is tightly controlled and requires massive changes at the mRNA level [15]. For example, the expression of many senescence-associated genes (SAGs) is initiated by upregulated levels of ROS [16,17]. Stress causes significant increases in jasmonic acid and ethylene levels [18]. The *Arabidopsis* mutants *ore1*, *ore3*, and *ore9* obviously have upregulated tolerance to various types of oxidative stresses and delayed leaf

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senescence, providing evidence that oxidative stress resistance is very important to control leaf senescence [19]. In addition, mutation analysis of transcription factors (TFs), such as AP2, RAV1, and RAP, shows that they have a regulatory role in the senescence of *Arabidopsis* [20]. Moreover, the expression of MYB, NAC, and WRKY TFs increases with increasing ROS levels [17]. *JUB1* negatively regulates senescence, most likely by affecting cellular H<sub>2</sub>O<sub>2</sub> homeostasis [21].

High concentrations of ROS can trigger programmed cell death. The hypersensitive response (HR) is the fastest defense reaction if contact is not inevitable [22]. The first reaction step is the oxidative burst, leading to local cell death around the infection site and thereby preventing the spread of a potential infection [23]; this is achieved through genes with stress-inducible promoters such as POD and alcohol dehydrogenase (ADH) [24]. Recently,  $\gamma$ -aminobutyric acid (GABA) shunt enzymes or genes have received increased attention because of their role in stress tolerance through the control of succinate levels and the accumulation of ROS [25].

In this study, the differences in gene expression between 3,000- and 20-year-old *P. orientalis* individuals were investigated, focusing on ROS production and clearance mechanisms, signal transduction, TFs, ROS scavengers, and stress resistance. The differential expression of ROS scavengers and stress resistance-related genes may play an important role in suppressing senescence in the 3,000-year-old *P. orientalis*. Our results provide data on the molecular mechanisms involved in senescence regulation and resistance in *P. orientalis* and may help improve the environments of *P. orientalis* and provide a theoretical foundation for the postponement of senescence.

## 2. Materials and methods

### 2.1. Plant materials

Fresh leaves were collected from the oldest *P. orientalis* tree, which is more than 3,000 years old (old tree) (three plots represent three biological replicates), and from three 20-year-old trees (adult tree) (three individuals for three replicates) as a control, which grow in similar conditions as the “Father” in Shaanxi, China (109°15′48.10″E, 35°35′8.28″N). Sun-exposed leaves were collected from the outside of the crowns with healthy new leaves in the first year and were found to be disease free. Approximately 5 g of fresh leaves were collected from biological replicates with similar heights and diameters at 8 am in August 2011 (under similar environmental conditions as much as possible). Leaves were flash frozen in liquid nitrogen and stored at –80°C.

Seedlings of *P. orientalis* were stressed and used for screening differentially expressed genes. Nine-month-old seedlings were treated with 200 mM NaCl and 150 mM ABA for 0, 12, 24, 48, and 72 h. Leaves were collected and immediately frozen in liquid nitrogen. Fresh leaves were collected from three seedlings to give three replicates.

### 2.2. Physiological Index determination

The levels of H<sub>2</sub>O<sub>2</sub>, malondialdehyde (MDA), glutathione (GSH), and GABA were measured according to Brennan and Frenkel [26], Heath and Packer [27], Ellman [28], and Shi et al. [9], respectively. The detection of superoxide radical (O<sub>2</sub><sup>-</sup>) was according to Wang et al. [29]. The measurement of hydroxy radical ( $\cdot$ OH) levels was performed according to Fenton Reaction (NJJCBIO, Beijing, China), whereas SOD, POD, and glutathione reductase (GR) activities were measured according to Jiménez et al. [30], Shaw [31], and Schaedle and Bassham [32], respectively. Vertical bars represent the mean  $\pm$  SD of four separate experiments. Data were analyzed by ANOVA in the SPSS software. Differences between different ages were considered statistically significant at  $P < 0.05$ .

### 2.3. Total RNA isolation

Total RNA was isolated from the leaves of *P. orientalis* according to the protocol of Column Plant RNAout kit (TIANDZ, Beijing, China) [33]. Then the quality and integrity of RNA was assayed using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and by electrophoresis through 1.5% agarose gels and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

### 2.4. Construction of a cDNA library and transcriptome sequencing

The RNA for transcriptome was obtained by mixing RNA extracts from 20- and 3000-year-old individuals of *P. orientalis*. Oligo (dT) magnetic beads were used to purify mRNA from 20  $\mu$ g of total RNA, and mRNA was broken into short segments and added to a fragmentation buffer. Short segments as templates with six-base random primers were used for first strand cDNA synthesis. Second strand cDNA synthesis used dNTPs, RNase H, and DNA polymerase I. Sequencing adapters were ligated onto the short segments after purification according to the protocol of QiaQuick PCR extraction kit (QIAGEN, CA, USA) for discriminating different sequencing samples. Segments were selected for PCR amplification and separated by agarose gel electrophoresis as sequencing templates. The Illumina GAllx system was used to complete the sequencing. The sequencing datasets were deposited in the NCBI database (Accession SRX1715098).

### 2.5. Transcriptome assembly and functional annotation

The raw reads of *P. orientalis* were filtered by removing adaptor sequences, empty reads, and low-quality sequences using Trinity [34]. Reads with N percentage over 10% and including more than 50% nucleotides in the read (Q-value#5) were also removed. Transcriptome assembly of *P. orientalis* was performed using the short read assembling software SOAPdenovo [35]. The longest transcript of *P. orientalis* in the clean reads was treated as a unigene. Functional annotations were performed by homology search against public databases using unigenes, such as Nr and Swiss-Prot, using BLASTx with a cutoff *E*-value of 10<sup>-5</sup>. Blast2 GO was applied to receive the relevant GO terms according to the Nr BLAST results, with a value <0.00001. Then, unigenes were used to query against the COG and KEGG databases to predict and classify functions and pathway assignments [36].

### 2.6. Analysis of differential gene expression tags

*P. orientalis* tag library preparation for these two age stages (20 and 3,000 years) was performed in parallel by using the Illumina gene expression sample preparation kit. Sequencing-received raw image data was transformed by base calling into sequence data. The sequences from the differential gene expression (DGE) analysis were available at the NCBI database (Accession SRX1755981). Before mapping these reads of *P. orientalis* onto the reference database, we filtered all sequences to eliminate empty tags, low-quality sequences and adaptor sequences, low complexity sequences, and tags with a copy number of 1 (possibly sequencing error). We calculated the number of expressed tags and then normalized to TPM (number of transcripts per million tags); DEGs were saved for another analysis.

### 2.7. Evaluation of DGE libraries

Statistical analysis of the frequency in each library was performed to count the expression ratios (fold change) between 20- and 3,000-year-old plants. The statistical comparison method was according to Audic [37]. The transcription abundance of each unigene was calculated through TPM. To identify the DEGs, a threshold false

discovery rate between (FDR)  $<0.001$  and  $|\log_2\text{Ratio}| \geq 2$  was used to identify the significant DEGs.

### 2.8. Quantitative real-time RT-PCR

RNA from 20- and 3,000-year-old *P. orientalis* leaves was extracted and purified as described above. Reverse transcriptase reaction was performed according to the protocol of PrimeScript™ RT reagent Kit (Takara, Dalian, China). qRT-PCR (Real-time quantitative PCR) result was detected according to the protocol of SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Dalian, China) in a 20- $\mu\text{L}$  volume containing 10  $\mu\text{L}$  SYBR® primer EX Taq (2 $\times$ ), 0.8  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), 2.0  $\mu\text{L}$  of eight-fold diluted cDNA template, and 7.2  $\mu\text{L}$  distilled water. The qRT-PCR program was as follows: 95°C for 10 s and 40 cycles of 95°C for 15 s and annealing at 60°C for 30 s. Products were analyzed from the melt curve at the end of amplification. The  $\alpha\text{TUB}$  was selected as the reference gene of *P. orientalis* [38]. Primers were designed using the Primer Premier 3.0 software. The final threshold cycle (Ct) values were calculated from three biological triplicates.

## 3. Results

### 3.1. ROS and scavenging systems in adult and old *P. orientalis*

As shown in Fig. 1, the contents of  $\text{H}_2\text{O}_2$ ,  $\text{O}_2^-$ ,  $\cdot\text{OH}$ , and MDA in the 3,000-year-old *P. orientalis* (the old tree) were 1.38-, 2.54-, 1.62-, and 1.28-fold ( $P < 0.05$ ) higher than those in the 20-year-old *P. orientalis* (the adult tree). The activities of GR and GSH showed the same trend, with the old tree showing 1.33- and 1.1-fold ( $P < 0.05$ ) higher activities than the adult tree, although the activity of SOD was decreased in the old tree. These results suggested that the extremely old *P. orientalis* still retained a stable ability to eliminate ROS, although some components of the ROS scavenging system were downregulated.

### 3.2. Transcriptome sequencing and de novo assembly

To obtain the gene expression of *P. orientalis* at different ages, cDNA samples were obtained from the 20- and 3,000-year-old individuals of *P. orientalis* and sequenced using the Illumina sequencing platform. In total, 1.2 billion 90-bp reads were assembled into 363,885 contigs

**Table 1**

Transcriptome data from *Platycladus orientalis* leaves.

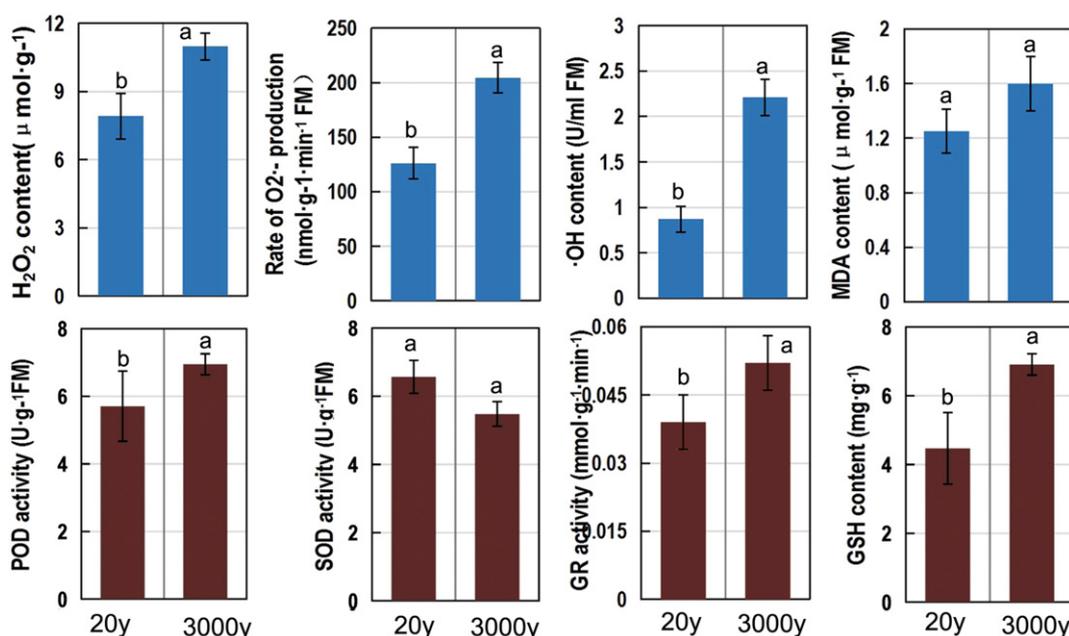
Total number of reads	13,600,516
Total base pairs (bp)	1,224,046,440
Average read length	90 bp
Total number of contigs	363,885
Mean length of contigs	142
N50	105
Total number of scaffolds	68,219
Mean length of scaffolds	389
N50	569
Total number of unigenes	51,664
Mean length of unigenes	475
N50	631

(Table 1). The average contig size was 142 bp, with a length range of 90–4647 bp. The contigs were further assembled into 68,219 scaffolds with an average size of 389 bp, comprising 7,929 scaffolds exceeding 1,000 bp. Then, 51,664 unigenes (scaffolds that matched no other scaffold) were generated from the 68,219 scaffolds, with an average size of 475 bp. The length distributions of contigs, scaffolds, and unigenes are shown in Table S1.

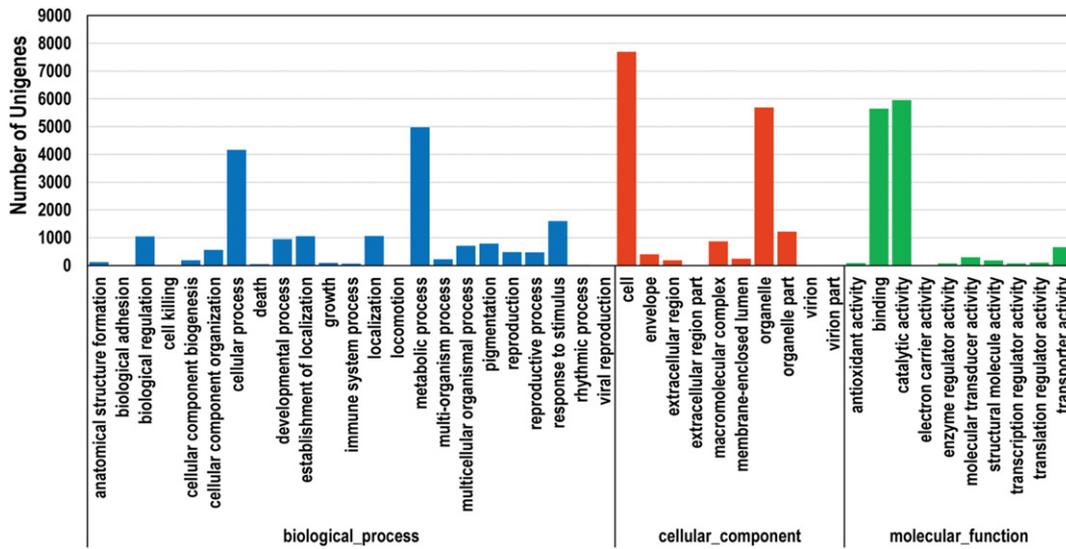
### 3.3. Functional classification of unigenes in *P. orientalis*

In this study, the 51,664 sequences were classified into 44 functional groups (Fig. 2). In the three main classifications of the GO, the “metabolic process,” “cell,” and “catalytic activity” terms were dominant. In addition, all the unigenes were classified according to the COG database. A total of 16,538 sequences were assigned COG classifications (Fig. 3). Among the 24 COG categories, the “general function prediction only” cluster (2,847, 17.21%) represented the largest group, followed by “transcription” (1,364, 8.25%) and “post-translational modification, protein turnover, and chaperones” (1,279, 7.73%), whereas only one unigene belonged to the “nuclear structure” group. Remarkably, 288 unigenes belonged to the ‘defense mechanisms’ group.

To identify the active biological pathways in *P. orientalis*, we analyzed all unigenes according to the KEGG database. In total, 15,031 unigenes belonged to 119 KEGG pathways (Table S2). Those unigenes belonged to “metabolism” (3745 unigenes, 24.92%), “secondary metabolism”



**Fig. 1.** Active oxygen molecules and the reactive oxygen species scavenging system in *Platycladus orientalis* leaves.



**Fig. 2.** Functional annotation of assembled sequences based on gene ontology (GO) categorization. We examined the main functional categories in the biological process, cellular component, and molecular function ontologies relevant to plant physiology. Bars represent the number of *Platyclusus orientalis* genes with BLASTX matches assigned to each GO term. One unigene could be matched to multiple GO terms.

(2282 unigenes, 15.18%), and “plant–pathogen interaction” (1211 unigenes, 7.46%). Moreover, the most represented ROS-related KEGG pathways were “peroxisome” (230 unigenes), “glutathione metabolism” (135 unigenes), and “ascorbate and aldarate metabolism” (126 unigenes).

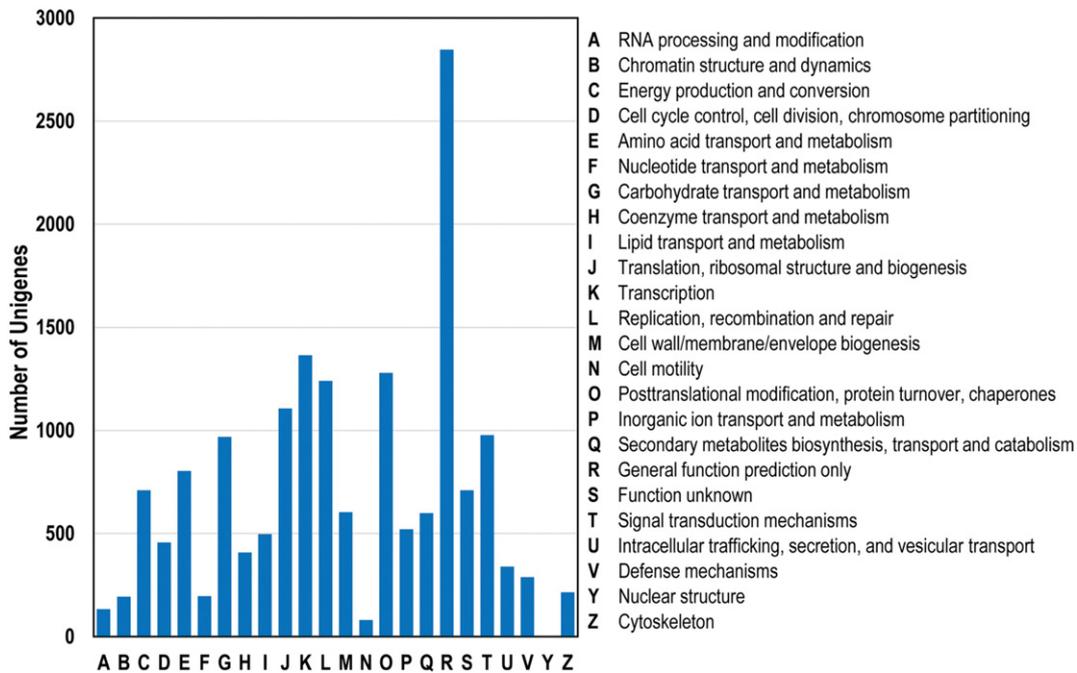
### 3.4. Identification of DEGs during senescence

To identify the key genes regulating the senescence process in the old tree and find the molecular mechanism of senescence resistance regulation, we examined the DEGs between the adult and old trees. A total of 106 DEGs were examined, including TF, hormone-related, ROS accumulation, senescence, and stress tolerance genes between the adult and old trees ( $FDR \leq 0.001$  and  $|\log_2\text{Ratio}| \geq 2$ ) (Fig. 4a, b; Table S3). qRT-PCR evaluation was then used to confirm the sequencing

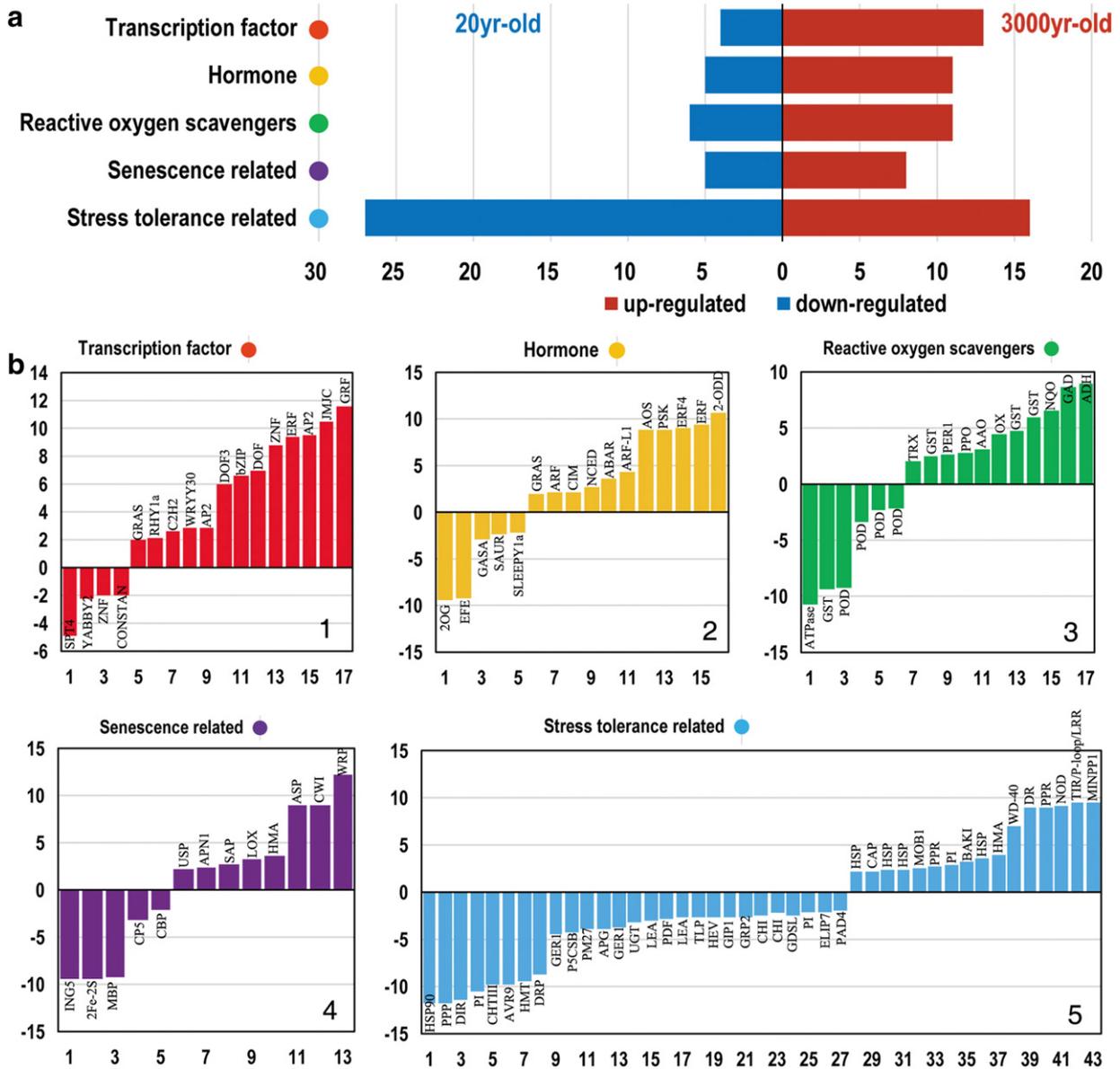
results. Eight genes were selected for this verification, including *WRKY*, *NADPH*, *POD*, *LOX*, *PAL*, *Cp5*, *CTK*, and *DRP* (Table S4). Their expression patterns were mostly consistent with the sequencing results (Fig. 5), which indicated that our DEG results were reliable.

### 3.5. DEGs belonging to TF families

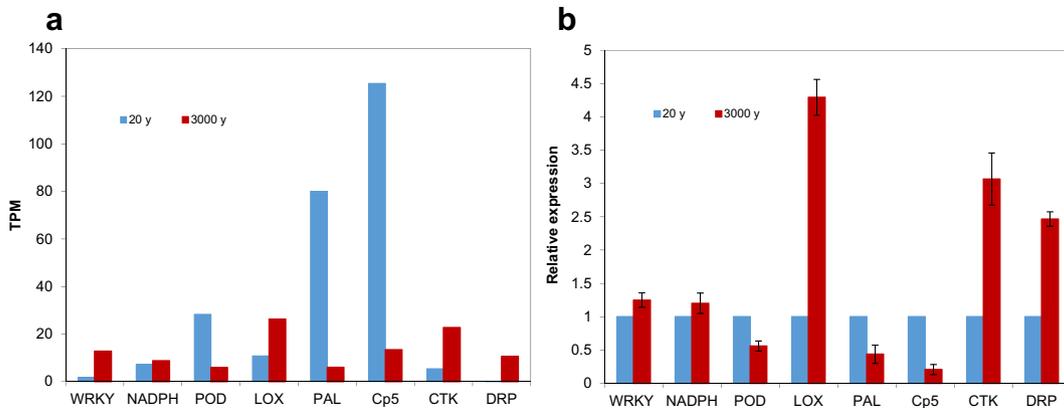
The coordinated regulation of gene expression during senescence depends on the combined action of several families of TFs. There were 12 upregulated and four downregulated TF genes in the old tree compared with the adult tree (Fig. 4b; Table S3); 8 of the 12 upregulated TF genes showed over five-fold increases in the old tree, including GRF zinc finger family, jumonji domain-containing, AP2 domain class, ethylene-responsive (ERF), and bZIP TF proteins. In addition, we found that some TFs related to stress tolerance were



**Fig. 3.** Clusters of orthologous groups (COG) classification. A total of 51,664 unigenes with Nr hits were grouped into 26 COG terms.



**Fig. 4.** Different expression genes (DEGs) and their expression levels in different functional categories. (a) A total of 106 DEGs were divided into five subgroups, namely transcription factors, hormone-related genes, reactive oxygen species scavengers, senescence-related genes, and stress response and defense genes. (b) The five subgroups of DEGs were classified further according to a  $FDR \leq 0.001$  and  $|\log_2 \text{Ratio}| \geq 2$ . Detailed information is shown in Table S3.



**Fig. 5.** qRT-PCR analysis of candidate genes in *Platyclusus orientalis*. Eight different expression genes were selected to verify the Illumina sequencing results by qRT-PCR.  $\alpha TUB$  was used as an internal control.

upregulated in the old tree, e.g., a zinc finger family protein and a GRAS family TF; these genes might play an important role in increasing its ability to resist senescence.

### 3.6. DEGs associated with hormones

Phytohormones regulate plant growth and development. In the present work, 11 genes associated with the ethylene, ABA, and JA hormones were upregulated and five genes were downregulated in the old tree compared with the adult tree (Fig. 4b; Table S3). The ethylene formation-related genes 2-oxoglutarate-iron oxygenase and ethylene-forming enzyme (EFE) showed 9.42- and 9.22-fold more expression, respectively, in the old tree. Conversely, a 2-oxoglutarate-iron oxygenase, a gibberellin-regulated family protein, and a SAUR family protein were downregulated in the old tree. This indicated that the old tree was growing slower.

### 3.7. DEGs involved in ROS scavenging

ROS and antioxidants play central roles in the regulation of senescence and other developmental processes [39]. Here, 17 ROS scavenging genes were differentially expressed between the adult and old trees, with 11 genes being upregulated and six genes downregulated (Fig. 4b; Table S3). The expression of ROS scavenging-related genes, such as *ADH*, glutamate decarboxylase (*GAD*) gene, NADPH:quinone oxidoreductase gene, and glutathione S-transferase (*GST*) gene, was upregulated in the old tree. In particular, *ADH* and *GAD* showed an increase of more than eight-fold, but genes including an AAA-type ATPase family protein gene, *GST*, and *POD* were downregulated. Overall, more ROS scavenging genes were upregulated in the old tree than in the adult tree.

### 3.8. DEGs involved in senescence and stress tolerance

In plants, the transition from juvenile through maturity to senescence is a physiologically complex process. In this study, 13 DEGs associated with senescence were identified (Fig. 4b; Table S3), six of which were upregulated in the old tree, including a wound-responsive family protein (*WRP*), cell wall invertase precursor, aspartic proteinase, and lipoxygenase. Among these, the *WRP* was significantly upregulated over 12.09-fold. The upregulation of these genes in the old tree indicated that extreme age induced clear upregulation of senescence-associated genes. Moreover, 43 DEGs associated with stress tolerance were identified, of which 16 genes were upregulated and 27 genes were downregulated in the old tree (Fig. 4b; Table S3). The upregulated genes included a TIR/P-loop/LRR gene, a nodulin gene, a disease resistance gene, and a WD-40 repeat protein-like gene. Among these, the nodulin gene's expression was increased by 9.21-fold in the old tree. The downregulated genes included an HSP gene, a pentatricopeptide repeat protein gene, a dirigent-like protein gene, and a dehydration-responsive family protein gene; among these, the *HSP* showed an 11.83-fold lower expression in the old tree compared with the adult tree. High expression of stress tolerance genes in the adult tree indicated that the tolerance capacity of the adult tree is higher than that of the old tree.

### 3.9. Expression patterns of ROS scavenging genes under NaCl and ABA

Antioxidant enzymes are crucial for the detoxification of ROS and the regeneration of cellular redox buffers (ascorbate and glutathione). In the present study, the expression of several antioxidant genes was obviously upregulated in the old tree. Seedlings of *P. orientalis* were stress treated and used for the identification of these genes. The *ADH*, *POD*, and GABA shunt genes were selected to further investigate their expression under ABA and NaCl treatments: *ADH* expression was highest at 24 and 48 h, respectively, while *POD* expression was lowest at 12 h and reached its highest peak at 24 h under these two stress factors (Fig. 6; Table S5). The expression of both *ADH* and *POD*

increased at first and then decreased. This suggested that *ADH* and *POD* coordinate cell stability and ROS scavenging under stress.

Of special importance are the GABA shunt genes, namely *GAD*, *GABA-T*, and *SSADH*, which have been found to have a close relationship with ROS accumulation under abiotic stresses in recent studies [9]. The expression of *GAD*, *SSADH*, and *GABA-T* in *P. orientalis* increased before 12 h and then decreased under NaCl and ABA stress, but *SSADH* expression was lower under NaCl than in the control (Fig. 6; Table S5). Correspondingly, the GABA content, catalyzed by *GAD*, increased under NaCl and ABA stresses, reaching its highest level at 6 and 48 h, respectively (Fig. 7). These results indicated that GABA shunt genes might play an important role against abiotic stress in *P. orientalis*.

During abiotic stresses, ROS accumulation depends greatly on the balance between ROS production and scavenging [40]. In this study, the  $H_2O_2$  content increased at first and then decreased with increasing NaCl treatment time (Fig. 7).  $H_2O_2$  accumulation reached its highest level at 48 h under NaCl stress and 24 h under ABA treatment. The  $H_2O_2$  content showed the same trend as the expression of the *ADH*, *POD*, and GABA shunt-related genes (*GAD*, *SSADH*, and *GABA-T*, respectively), suggesting that plant cells can maintain homeostasis between the formation and removal of ROS through particular enzymatic pathways or antioxidants.

## 4. Discussion

Stresses induce the accumulation of high amounts of substances that are harmful to cells. If not promptly eliminated, these substances cause damage to the cell membrane or other organelles [9]. There were obviously negative correlations between the contents of GR, GSH, and  $H_2O_2$  in 20- and 3,000-year-old *P. orientalis* in the current study. Enzymatic ROS scavenging enhances the ability to eliminate ROS, which may be the primary physiological mechanism for slowing senescence or increasing longevity. ROS accumulation, the physiological cause of senescence and death, was higher in the adult tree than in the old tree in this study, which may result from the effect of individual and environmental factors over the years [1].

The molecular mechanism that the 3,000-year-old *P. orientalis* has adapted to a variety of stresses and resists senescence requires further study. We used a high-throughput mRNA sequencing technology (Solexa) to generate 51,664 unigenes with an average length of 475 bp. Functional annotation and expression profile analysis of all these unigenes revealed that most genes had low or moderate expression abundance in the transcriptome, which is similar to the results of *Siraitia grosvenorii* [36]. The quality of transcriptome in this study was relatively low compared to the transcriptome of *P. orientalis* in another study [41] because our data was collected in 2011. However, the present transcriptome data can provide a gene resource for the further investigation of adaptation to external stresses and diseases in the old *P. orientalis*.

The identified genes were grouped into six functional classes, as shown in the pie chart based on the MetaCyc, KOG, and GO databases. DGE analysis showed that TFs-, hormone-, antioxidant-, and senescence-related genes were clearly upregulated in the old tree. In particular, we found that TFs related to senescence, such as GRFs, bZIP, and WRKY TFs, were upregulated in the old *P. orientalis* tree. bZIP TFs play crucial roles in defending against pathogens [42]. WRKY30 is increased markedly during the leaf senescence process, and both WRKY53 and WRKY30 have been shown to be responsive to ROS [43]. The mRNA levels of certain TFs are increased greatly during the senescence process in *Arabidopsis* and *Populus* [44,45]. These TFs might play an important role in increasing the resistance of the old tree. However, further identification and characterization of TFs in *P. orientalis* is needed in the future.

The expression of genes regulated by ethylene was increased in the old tree, e.g., 2-*ODD*, which participates in ethylene biosynthesis, an

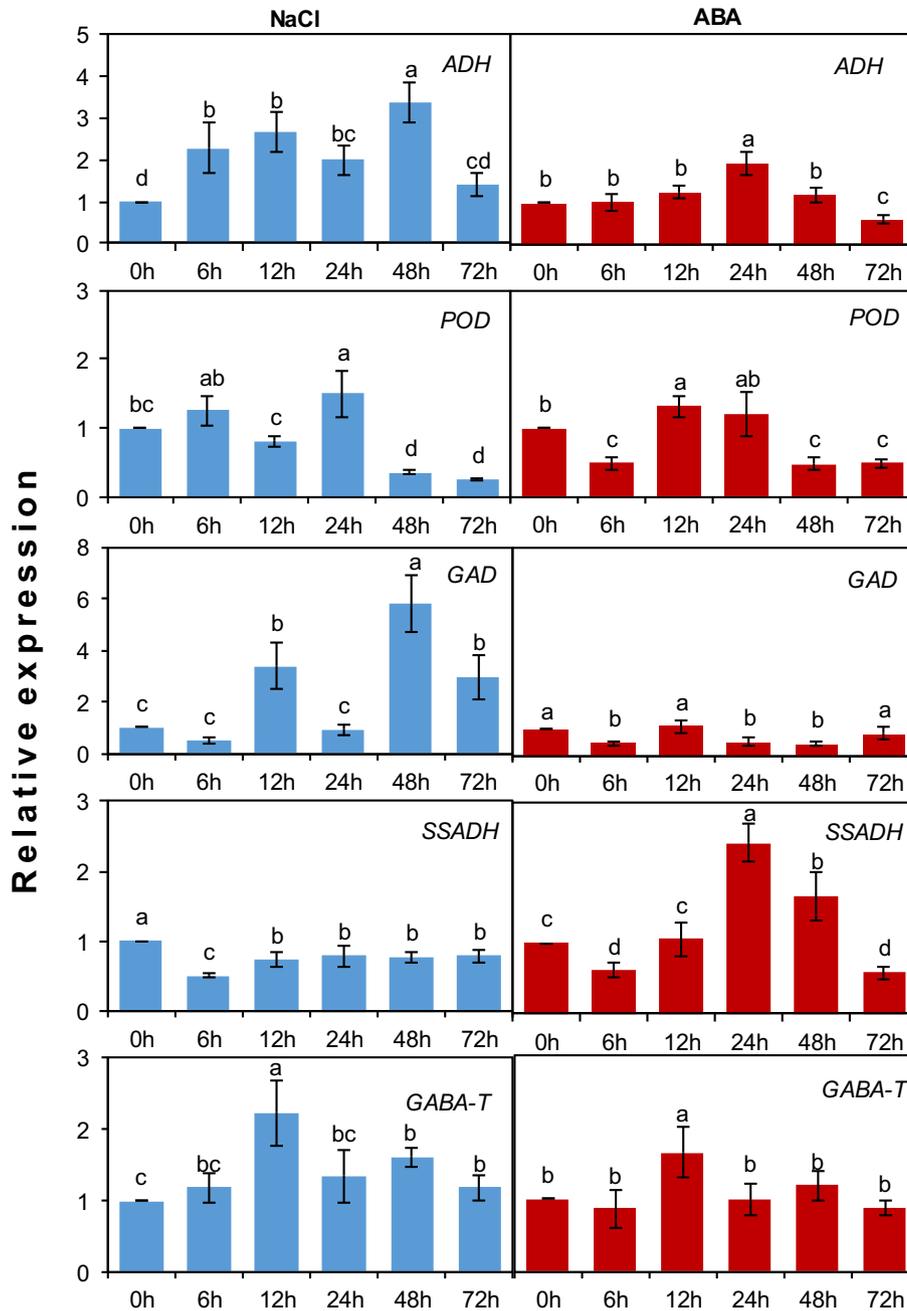


Fig. 6. Expression patterns of reactive oxygen species scavenging-related genes in the seedlings of *Platycladus orientalis* under exogenous ABA and NaCl stress.

*ERF* belonging to the ERF subfamily of the AP2 family, which is unique and was first identified as an EREBP (ethylene-responsive element binding protein), and *ERFS*, which activates the expression of resistance- and pathogenesis-related proteins [46,47] such as osmotin, P-1,3-glucanase, and chitinase [48,49]. ABA triggers the production of ROS including  $H_2O_2$  in guard cells, which in turn promotes stomatal closure [50]. Our results showed that the old tree is growing slower; therefore, the hormonal mechanism in older trees needs to be studied further.

Most ROS scavenging genes were upregulated in the old tree, such as ADH genes, which are among the most common cold-induced genes in cereal crops and *Arabidopsis* [51]. The detoxification and antioxidant activity of GSTs is considered an important aspect of the stress resistance of barley genotypes but requires further investigation. Notably, the expression of glutamate receptor 1 gene and *GAD* was significantly increased in the old tree. *GAD* activity induced GABA

synthesis in germinated *Glycine max* under hypoxia treatment [52]. A homolog of *GAD* is required for ROS stress resistance in yeast, and under stress, the *gad1* mutation induced GABA accumulation and prevented ROS accumulation and cell infection in plants [53]. In general, increased expression of ROS scavenger genes in the old tree probably increases its ability to resist stress. These important genes were reported for the first time in *P. orientalis*.

Recent gene function research has identified many senescence-associated genes (SAGs) that are differentially expressed during plant senescence [17]. Protein degradation is one of the most remarkable characteristics of plant senescence. Aspartic proteinases participate in the processes of apoptosis and programmed cell death [54]. *AtLOX1* has enhanced expression during senescence [55,56]. The upregulation of these genes in the old tree compared with the adult tree was consistent with its senescence state. However, the expression of SAGs in *P. orientalis* of different ages needs to be studied further.

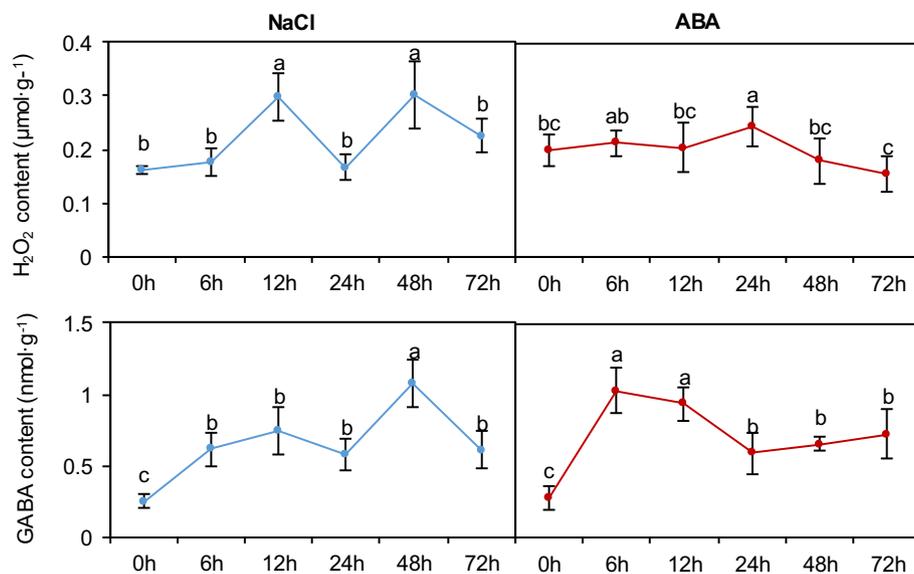


Fig. 7. GABA and H<sub>2</sub>O<sub>2</sub> content of the seedlings of *Platycladus orientalis* under exogenous ABA and NaCl stress.

HSP and DIR are stress tolerance-related genes. HSPs are highly conserved proteins that facilitate the assembly, maturation, stabilization, and activation of important signaling proteins such as TFs, hormone receptors, and protein kinases in plant cells [57]. Pentatricopeptide proteins can regulate ROS homeostasis and stress-induced ABA responses [58], while DIR can promote resistant substances for the protection of plants from fungal infections and diseases [59]. Chitinases play a very important role in active or passive defense against pathogens. Higher expression of these genes corresponds to better stress tolerance of the adult tree compared with the old tree. The introduction and expression of these genes make plants resistant to diseases and improve their resistance to pathogens, but the regulation of the expression of these genes has not been studied in *P. orientalis*.

Plants under stress accumulate substantial ROS through cellular metabolism that requires prompt removal. *P. orientalis* seedlings under NaCl and ABA stress showed increased levels of *POD* and *ADH* expression. *POD* transcription helps remove intracellular ROS and improves plant disease resistance through the oxidation of phenol into quinone [60], while *ADH* genes are among the most common cold-induced genes in cereal crops and *Arabidopsis* [51]. GABA as an exogenous substance in *Caragana intermedia* under salt treatment can inhibit the accumulation of H<sub>2</sub>O<sub>2</sub> and regulate the expression of TFs-, hormone metabolism-, and ROS-related genes, such as the NADPH oxidase gene *RBOH* [9]. Here, the GABA and H<sub>2</sub>O<sub>2</sub> contents show the same trend; thus, our experimental results agree with the results of theoretical analysis. GABA can be a nitrogen source or a signal molecule, but its relationship with ROS has attracted the most attention [61]. In summary, the old tree maintained a high growth capacity and high activity of ROS scavenging enzymes. ROS, as the main factor affecting senescence, might be able to adjust the control of or influence on senescence, which has also become a hotspot of research in the field of resistance to senescence in trees in recent years [62].

## 5. Conclusion

Biochemical and physiological indices both suggested that the old tree had great antioxidant activity in the present study. Excluding unknown genes, our gene expression data showed that ROS scavenging genes and related TFs were clearly induced in the old tree, while the upstream

regulation of genes, such as ethylene- and senescence-related genes, showed that the old tree might be in a senescent state. The activities of ROS scavenging enzymes were higher in the old tree. Moreover, the enhanced expression of the GABA shunt-related gene *GAD* in the old tree indicated that this shunt has a special role in resisting stresses. Thus, studying these genes could improve our knowledge of anti-senescence mechanisms, and stress resistance-related genes could be used in transgenic approaches in ornamental plant breeding to improve stress resistance.

## Conflict of interests

There is no conflict of interest.

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## Supplementary data

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