Signal Molecules Crosstalk and the Critical Role of Jasmonic Acid in Triterpenoid Synthesis Inducement of *Aspergillus Niger* Elicitor in Suspension Cultured *Cyclocarya Paliurus* Cells

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Abstract

Background: *Cyclocarya paliurus* (*C. paliurus*) leaves contain multiple health beneficial metabolites, and therefore are often used in functional foods and Chinese herbal medicines. However, breeding difficulties confines its utilization. Consequently, cell suspension culture was developed to produce the bioactive secondary metabolites of *C. paliurus* leaves, but the content was comparatively low. In the present paper, *Aspergillus Niger* Elicitor (ANE) was used to stimulate the synthesis of triterpenoids in the suspension cultured *C. paliurus* cells, and the signal molecules crosstalk involved in this elicitation was further studied to interpret the underlying mechanism.

Results: Total triterpenoids accumulation of the cultured *C. paliurus* cells elicited by 200 µg/mL ANE was 9.17 times higher than that of the untreated cells. Nitric oxide (NO), hydrogen peroxide (H₂O₂) and jasmonic acid (JA) played important roles in the elicitation as signal molecules. Under the ANE stimulation, the concentrations of NO, H₂O₂ and JA all increased significantly, but presented different change profiles and peaked at different times. Based on series experiments of NO quenching by C-PTIO, H₂O₂ blocking by DMTU, and JA synthesis inhibition by IBU and NDGA, together with exogenous NO, H₂O₂ and JA addition experiments, it was deduced that ANE improved triterpenoids synthesis in the suspension cultured *C. paliurus* cells via a complex signal transduction network, in which three deduced and three hypothetical signal transduction pathways might be involved. JA was not only the junction of NO and H₂O₂ signal pathways, but also the critical point in the whole signal network. RNA-seq analysis showed that a total of 3 candidate JA synthesis pathway genes including 1 *LOX* and 2 *OPR* were found to be significantly up-regulated under the ANE stimulation, along with 5 down-regulated *JAZs* and 1 up-regulated *JAR1* regulating to JA signal transduction.

Conclusions: ANE can significantly increase the triterpenoids synthesis in the suspension cultured *C. paliurus* cells. RNA-seq analysis validated the pivotal role of JA in this ANE elicitation. Our results provided references for the further studies on triterpenoids synthesis in *C. paliurus* under ANE treatment.

Background

Cyclocarya paliurus (Bata1.) Iljinskaja (*C. paliurus*) is a well-known edible and medicinal herb belonging to the genus of Juglandaceae, which mainly grows in the south of China [1]. Its leaves contain multiple kinds of bioactive compounds such as triterpenoids [2], flavonoids [3], polysaccharides [4] and polyphenols [5], and therefore display various bioactivities including anti-tumor [6], regulating lipid metabolism [7] and anti-diabetes [2]. Unfortunately, *C. paliurus* is on the verge of extinction due to difficulties on the reproduction and cultivation, which dramatically limits its use in medicines and foods, and also drives researchers to develop new techniques to better utilize this resources. Plant cell culture technology presents many advantages on the production of plant secondary metabolites, including short production cycle, better controllability, and independently of climate and weather [8], and therefore it is regarded as a feasible method to make full of the *C. paliurus* resources.

A series of researches focused on the *C. paliurus* resources utilization had been carried out in our laboratory, such as metabolites identification and quantification [9, 10], metabolites bioactivity [11–13], callus inducement and screening [14] and cell suspension culture [15, 16]. Now, a stable cell suspension culture had been established to produce the main bioactive components of *C. paliurus* in our laboratory.

Triterpenoids of *C. paliurus* have been attracting the researcher's attention for its excellent bioactivities [17]. Eight triterpenoids, including arjunolic acid, betulinic acid, corosolic acid, maslinic acid, oleanolic acid, ursolic acid, β -amyrin and β -boswellic acid, were identified from the leaves of *C. paliurus* in our previous studies [10]. Among them, five had been found in our suspension cultured *C. paliurus* cells [18]. To further improve the triterpenoids yield, sodium nitroprusside was applied into the cultured medium as an abiotic elicitor, and the results showed that total triterpenoids yield of the elicited cells was 2.14 times that of the control group [19].

Elicitation is one of the most effective ways to stimulate the secondary metabolite synthesis in the plant cell, and therefore is now widely employed to induce novel metabolites or enhance the accumulation of the target metabolites in the plant cell, tissue and organ cultures [20]. According to chemical property, the elicitors are normally classified into two types, including abiotic elicitors (e.g. methyl jasmonate, salicylic acid (SA), AgNO_3 and CaCl_2) and biotic elicitors (e.g. fungal extracts, bacterial extracts and chitin) [21, 22]. These elicitors can trigger defense or stress-induced responses at a low concentration, which can further regulate the expression level of some biosynthesis pathway genes via complex signal transduction networks and finally promote the synthesis of secondary metabolites [20]. Fungal extract is one of the most effective and studied elicitors in the last three decades. Kümmeritz et al [23] reported that adding fungal medium filtrates combined with sucrose feeding significantly enhanced triterpenes content of *Salvia fruticosa* cell suspension culture by approximately 140%, and the triterpenes yield almost reached the level of intact plants. According to Li et al., the glycyrrhetic acid content of the cultured *Glycyrrhiza uralensis* adventitious roots raised 1.8 times with the addition of *Aspergillus niger* elicitors (ANE) [24]. However, up to now the mechanism of ANE elicitation is not well understood, especially the involved signal transduction network.

In the present paper, ANE was used as a biotic elicitor to increase the triterpenoids synthesis in the suspension cultured *C. paliurus* cells. To interpret the elicitation mechanism, the profile changes of four signal molecules concentration were determined in detail after ANE inducing, meanwhile, the crosstalk among these signal molecules was further studied by a series of experiments of signal molecules blocking, as well as experiments of exogenous signal molecules addition. What's more, differentially expressed genes (DEGs) associated with the jasmonic acid (JA) synthesis and transduction were also analyzed to demonstrate the critical role of JA in this elicitation by RNA-seq. To the best of our knowledge, at present there is no report on the signal molecules crosstalk involved in the ANE elicitation in the suspension cultured *C. paliurus* cells. Our results and analysis provided references for the further studies on the triterpenoids biosynthesis regulation of the cultured plant cells.

Materials And Methods

Materials

Suspension cultured cell lines of *C. paliurus* was cultivated and maintained in Jiangxi Key Laboratory of Natural Products and Functional Food (Jiangxi Agricultural University, Nanchang, China).

Aspergillus niger (CGMCC3.0453) was purchased from China General Microbiological Culture Collection Center (CGMCC).

Maslinic acid ($\geq 98\%$), corosolic acid ($\geq 98\%$), betulinic acid ($\geq 98\%$), oleanolic acid ($\geq 98\%$) and ursolic acid ($\geq 98\%$) were all purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China).

Cell suspension culture

According to the method reported by Yin et al. [9], *C. paliurus* cells were cultured in a basal liquid MS medium (Murashige and Skoog) supplemented with 2, 4 - D (2 - (2, 4 - dichlorophenoxy) acetic acid, 0.5 mg/L), NAA (1 - Naphthaleneacetic acid, 0.3mg/L) and KT (6 - Furfurylaminopurine, 1.0mg/L) in the dark at $25 \pm 1^\circ\text{C}$ on a rotary shaker at 115 rpm, and sub-cultured every 6 days.

Aspergillus niger elicitor preparation

ANE (*Aspergillus niger* elicitor) was prepared according to the method of Xiong et al. [25].

Elicitation method of ANE on the cultured *C.paliurus* cells

Elicitation was performed in accordance with the method described by Xiong et al. [25]. ANE at a concentration of 200 $\mu\text{g/mL}$ was added into the culture medium on the 4th day after cell inoculation to stimulate the synthesis of triterpenoids. The cultured cells were respectively sampled at 0th, 20th, 40th, 60th and 80th hour after ANE elicitation for triterpenoids and signal molecules determinations.

Triterpenoids extraction and determination

Triterpenoids were extracted by ultrasound wave [18]. A total of 0.5 gram fine powder sample was dispersed in 6ml ethyl acetate and extracted in an ultrasonic extractor at 60°C for 30min. After centrifugation at 4000 r/min for 10 min, the supernatant was taken out and volatilized to complete dryness at 60°C , then redissolved in methanol for triterpenoids determination. The triterpenoids determination was carried out according to the HPLC method of Yin et al. [9]. Total triterpenoids content was the sum content of maslinic acid, corosolic acid, betulinic acid, oleanolic acid and ursolic acid.

Signal molecules assay

A total of four signal molecules, including nitric oxide (NO), hydrogen peroxide (H_2O_2), Jasmonic acid (JA) and salicylic acid (SA), were assayed by enzyme-linked immunosorbent assay method in the present paper. NO assay kit was purchased from Beyotime Bio-Technology Co., Ltd (Shanghai, China). H_2O_2 , JA

and SA assay kit were all provided by Yihan Bio-Technology Co., Ltd (Shanghai, China). All determinations were performed according to the instruction of assay kits.

Quenching of NO

C-PTIO (Enzo life Sciences, Switzerland), a strong quencher of NO, was used to eliminate NO in the cell cultures [26]. 20 minutes before the addition of ANE, C-PTIO solution was put into the culture medium, and the final C-PTIO concentration was controlled at 0.1, 1 and 100 μM respectively. The content of triterpenoids, H_2O_2 , JA and SA in the cultured cells were respectively determined when the NO was quenched by the added C-PTIO.

Blocking of H_2O_2

DMTU (Sigma-Aldrich, USA) was used as H_2O_2 scavenger in this paper [27]. 20 minutes before the ANE elicitation, DMTU (final concentration of 0.5, 1 and 2 mM were set) was added into the culture medium. The changes of triterpenoids accumulation were tested as well as the concentration changes of NO, JA and SA content when H_2O_2 were blocked by DMTU, Which were served to explore the role of H_2O_2 in triterpenoid synthesis elicitation of ANE.

Inhibition of JA synthesis

Two inhibitors, named IBU and NDGA, were conjointly used to block JA synthesis in the cultured *C. paliurus* cells [28,29]. Three combinations of IBU and NDGA concentration were applied into the culture medium 20 minutes before the ANE elicitation in this paper, including 25 μM IBU and 25 μM NDGA, 50 μM IBU and 50 μM NDGA, and 100 μM IBU and 100 μM NDGA, which was served to investigate the role of JA in the triterpenoid synthesis stimulation of ANE.

Exogenous addition of signal molecules

Sodium nitroprusside (SNP, Sigma-Aldrich, USA, 99%) was used as exogenous NO donor to activate NO signal pathway [30]. Methyl jasmonate (MJA, Sigma-Aldrich, USA, 95%) was applied as JA donor to generate JA signal molecules [31]. SNP, MJA and H_2O_2 (Xilong Chemical Co., Ltd. Guangdong, China, 30%) were respectively added into the *C. paliurus* cells culture medium at the same time of ANE elicitation as positive signal molecules control group, whose final concentrations were set at 150 μM , 10 μM and 50 μM respectively

RNA extraction and sequencing

The cultured cells were harvested at 20th hour after ANE elicitation and immediately frozen in liquid nitrogen and stored at -80 °C for the following total RNA extraction, as well as the untreated cells. Total RNA was extracted from the cultured *C. paliurus* cells using plant RNA purification reagent (Invitrogen, Carlsbad, CA, USA). The quantity and quality of total RNA were determined by Nanodrop 2000 (Thermo Fisher, America) and Agilent 2100. High-quality total RNA used for RNA-seq should have a concentration

greater than 200 ng/μL, and the RNA integrity number (RIN) must reach 7.5, meanwhile OD 260 /280 should be between 1.8 and 2.2. Poly (A) mRNA was enriched from total RNA using Oligo (dT) magnetic beads. The enriched mRNA was randomly broken into short fragments and then was used to synthesize the first-strand cDNA using reverse transcriptase and random primers, which was further served to synthesize the second-strand. These synthesized cDNA fragments were purified and dissolved in EB buffer for end repairing and adding of ploy (A), and then connected with sequencing adapters. The suitable fragments were selected for PCR amplification as templates to establish cDNA library. Finally the cDNA library was sequenced using an Illumina HiSeq 4000 platform with the 2×151 bp paired-end reads.

Reads alignment and De novo assembly

The raw reads, which were transformed from base calling file, were filtered out to generate clean reads by removing adaptor sequences, ambiguous reads and the low quality reads (quality value <20). The clean reads were assembled into transcript contigs using the short reads assembling program Trinity software (<http://trinityrnaseq.sourceforge.net/>, release-20140413), then the contigs were further connected until it could not be extended on either end, which were defined as unigenes.

Function annotation and classification of unigenes

All assembled unigenes were annotated using BLASTx alignment (e-value<0.00001) against the following publicly available protein databases: NCBI non-redundant (NR), Swiss-Prot, Pfam, eukaryotic orthologous groups (COG) and KEGG. Combining NR annotation with the Blast2GO program [32], GO annotations of the unigenes were obtained. All GO functional classifications were produced by WEGO software [33]. In the meantime, the pathway assignments were performed in conjunction with the KEGG database. When a conflict occurred among the database results, a priority order of alignments was Nr, Swiss-Port, KEGG, GO and COG.

Identification of differentially expressed genes

Normalized expression levels of the unigenes were calculated as fragment per kilobases of exon model per million mapped reads (FPKM) by the RSEM software [34]. The differential expression analysis of any two groups was analyzed using the EdgeR software and false discovery rate (FDR) method [35]. Afterward, the significantly differentially expressed genes (DEGs) were identified by the threshold of $FDR < 0.05$ and $\log_2|\text{Fold Change}| \geq 1$. To further identify significantly over-represented metabolic pathways or signal transduction pathways, all DEGs were mapped to KEGG database with a threshold E-value of 10^{-5} for pathway enrichment analysis.

Results

Stimulation effects of ANE on the triterpenoids synthesis in the cultured cells

To further enhance the triterpenoids production, ANE, a widely used biotic elicitor, was applied to stimulate triterpenoids synthesis in the cultured cells. Our results (as showed in Fig.1) indicated that the accumulation of triterpenoids in the cultured *C. paliurus* cells enhanced significantly under the elicitation of ANE at a concentration of 200 µg/mL, while the biomass was unaffected. With the time increasing, the yield of total triterpenoids rose first and fell later, and peaked at 60 hours after the ANE addition. The peak total triterpenoids yield of the elicited cells was 9.17 times higher than that of the untreated cells. However, ANE's elicitation mechanism is still unclear. To better understand the mechanism, four signal molecules crosstalk was further investigated as well as the RNA-seq.

Profile changes of four signal molecules concentration under the ANE elicitation

After the elicitation of 200 µg/mL ANE, the concentrations of NO, H₂O₂ and JA all increased significantly in the cultured *C. paliurus* cells, but presented different change profiles and peaked at different time (as shown in Fig. 2(a-c)). While, SA concentration fell gradually at the beginning, then kept relatively stable and lower than that of the untreated cells until 80 hours post-elicitation (Fig. 2(d)). Consequently, it was deduced that NO, H₂O₂ and JA was involved in the signal transduction of ANE elicitation as important signal molecules.

NO concentration rise is a common physiological reaction in plant cells when stimulated by elicitors (Xu et al., 2005). Our results (Fig. 2(a)) showed that NO concentration increased swiftly after the addition of ANE, and peaked at the 15th minute, then decreased sharply and maintained at a similar level of the un-elicited cells from 4 to 80 hours post-elicitation. Similarly, H₂O₂ concentration also enhanced rapidly under the ANE stimulation, and also reached a high concentration at the 15th minute (Fig. 2(b)). Nevertheless, unlike NO, H₂O₂ concentration always kept at a high level from 15 minutes to 80 hours, which was significantly higher than that in the untreated cells (Fig. 2(b)). The concentration changes of JA lagged behind that of NO and H₂O₂ (Fig. 2(a-c)). Within 6 hours after ANE elicitation, JA concentration was almost the same as that in un-elicited cells. From the 7th to 80th hour post-elicitation, JA concentration was obviously higher than that of control group, with two peaks appearing at the 18th and 42nd hour after ANE addition respectively (Fig. 2(c)). The highest concentration of JA occurred at the 42nd post-elicitation. In view of the time sequence changes of these signal molecules, a series of experiments of NO quenching by C-PTIO, H₂O₂ blocking by DMTU, and JA synthesis inhibition by IBU and NDGA were further performed to understand the crosstalk among these signal molecules, together with exogenous NO, H₂O₂ and JA addition experiments.

Changes causing by NO quenching and exogenous addition

As shown in Fig.3(a and b), NO concentration decreased gradually with the C-PTIO concentration increasing from 0.1 to 100 µmol/L, and fell to the level of the untreated cells at 100 µmol/L, but the C-PTIO addition and NO change had no influence on the cell growth (biomass was given in Fig. 3(b)). When 100 µmol/L C-PTIO was applied to the cultured cells elicited by 200 µg/mL ANE, H₂O₂ concentration was

approximately the same as that in the elicited cells, while JA concentration declined significantly, but still obviously higher than that of control group (neither ANE nor C-PTIO was added) (Fig. 3(c and d)). With NO blocked by C-PTIO, triterpenoids yield of the ANE elicited cells reduced by 21.28% percent, but still markedly higher than that of the control group (Fig. 3(e)).

To further interpret the role of NO and its relationship with H_2O_2 and JA, 150 $\mu\text{mol/L}$ SNP (exogenous NO donor) was added into the culture medium of *C. paliurus* cells. Results (Fig. 3(f and g)) indicated that exogenous NO showed no effect on H_2O_2 concentration, while raised the concentration of JA significantly. However, JA concentration in the cells treated by exogenous NO was still lower than that in ANE elicited cells. Under the exogenous NO stimulation, triterpenoids yield was 4.86 times that of the control group, but significantly lower than that of the ANE treated cells (Fig. 3(h)).

Based on the above analysis, the following three conclusions were obtained: firstly, ANE elicitation promoted the synthesis of triterpenoids in *C. paliurus* cells partly through NO signal pathway; secondly, JA was involved in NO pathway in ANE elicitation, and located in downstream of NO pathway; thirdly, H_2O_2 synthesis might be independent on the change of NO concentration.

Changes causing by H_2O_2 blocking and exogenous addition

As DMTU concentration rose from 0.5 to 2 mmol/L, the elimination rate of H_2O_2 , increased in the *C. paliurus* cell cultures accordingly (Fig. 4(a)). Under the scavenging of 2 mmol/L DMTU, H_2O_2 concentration in the ANE elicitation cells fell to the level of the un-elicited cells. Meanwhile JA concentration reduced by 14.53% percent, but still obviously higher than that in the control (Fig. 4(c)). 2 mmol/L DMTU showed no significant impact on cell growth ((Fig. 4(b))), but decreasing the triterpenoids yield of the ANE treated cells by 13.76% percent (Fig. 4(d)). Although triterpenoids accumulation in ANE elicited cells was reduced by 2 mmol/L DMTU, but still higher than that in the control group (Fig. 4(d)).

When exogenous 50 $\mu\text{mol/L}$ H_2O_2 was added into the culture medium of *C. paliurus* cells, JA concentration increased by 11.31% percent correspondingly, but was still significantly lower than that of the ANE elicited cells (Fig. 4(e)). Stimulated by 50 $\mu\text{mol/L}$ H_2O_2 , triterpenoids accumulation rose by 78.35 % percent, however, it was notably lower than that in the ANE treated cells (Fig. 4(f)).

In conclusion, ANE elicitation improved triterpenoids synthesis in *C. paliurus* cells partly through H_2O_2 signal pathway, and the change of H_2O_2 concentration could cause the change of JA concentration and finally led to a fluctuation of triterpenoids yield accordingly.

Changes causing by JA synthesis inhibition and exogenous JA addition

The combined application of IBU and NDGA could effectively as inhibit JA synthesis in the cultured *C. paliurus* cells. With the addition of 100 $\mu\text{mol/L}$ IBU and NDGA, JA concentration in the ANE elicited cells declined to the level of the un-elicited cells (Fig.5(a)), while the cell growth was unaffected (Fig.5(b)). Under the inhibition of 100 $\mu\text{mol/L}$ IBU and NDGA, triterpenoids yield reduced to 16.69 mg/40mL, which

was 59.41% percent of the ANE treated cells, but still significantly higher than that of the control (Fig.5(c)). When 10 $\mu\text{mol/L}$ MJA was used as JA donor in the culture medium, triterpenoids yield improved to 24.33mg/40mL, which was 4.92 times that of the control (Fig.5(d)).

Comparatively speaking, JA blocking resulted in the biggest decline of triterpenoids yield in the ANE elicited *C. paliurus* cells (Fig.5(c)), followed by NO and H_2O_2 blocking (Fig.3(e) and Fig.4(d)). Similarly, exogenous JA addition led to the highest increase of triterpenoids yield in the cultured *C. paliurus* cells (Fig.5(d)), followed by NO and H_2O_2 addition (Fig.3(h) and Fig.4(f)).

Consequently, it was deduced that JA was the critical signal molecule for triterpenoids yield promotion in the ANE elicitation, and the change of JA concentration could cause a notable change of triterpenoids accumulation in the culture *C. paliurus* cells.

Summary of Signal molecules crosstalk and deduced signal transduction pathways involved in ANE elicitation

Our signal molecules blocking and exogenous addition experiments suggested that NO, H_2O_2 and JA were all involved in the responses of ANE elicitation in the suspension cultured *C. paliurus* cells; the blocking of any one of NO, H_2O_2 and JA led to triterpenoids accumulation decline in the ANE elicited cells. Conversely, exogenous addition of any one of NO, H_2O_2 and JA resulted in triterpenoids synthesis rising, but their induced increments were all lower than that of ANE; both NO and H_2O_2 had apparent impacts on JA synthesis; the change of NO concentration showed no effect on the H_2O_2 synthesis; the influence of JA on triterpenoids synthesis was relatively more significant than that of NO and H_2O_2 . Based on the above analyses, three signal transduction pathways were deduced, which were shown in Fig. 6 and described as following: (1) ANE $\rightarrow$ receptor $\rightarrow$ nitric oxide synthase (NOS) $\rightarrow$ NO $\rightarrow$ jasmonic acid synthesis enzymes $\rightarrow$ JA $\rightarrow$ TFs $\rightarrow$ triterpenoids synthesis enzymes $\rightarrow$ triterpenoids; (2) ANE $\rightarrow$ receptor $\rightarrow$ NADPH oxidase $\rightarrow$ H_2O_2 $\rightarrow$ jasmonic acid synthesis enzymes $\rightarrow$ JA $\rightarrow$ TFs $\rightarrow$ triterpenoids synthesis enzymes $\rightarrow$ triterpenoids; (3) ANE $\rightarrow$ receptor $\rightarrow$ NADPH oxidase $\rightarrow$ H_2O_2 $\rightarrow$ nitric oxide synthase (NOS) $\rightarrow$ NO $\rightarrow$ jasmonic acid synthesis enzymes $\rightarrow$ JA $\rightarrow$ TFs $\rightarrow$ triterpenoids synthesis enzymes $\rightarrow$ triterpenoids. In addition, there might be another three hypothetical signal transduction pathways involved in ANE elicitation, which need to be further validated: (1) ANE $\rightarrow$ receptor $\rightarrow$ nitric oxide synthase (NOS) $\rightarrow$ NO $\rightarrow$ TFs $\rightarrow$ triterpenoids synthesis enzymes $\rightarrow$ triterpenoids; (2) ANE $\rightarrow$ receptor $\rightarrow$ NADPH oxidase $\rightarrow$ H_2O_2 $\rightarrow$ TFs $\rightarrow$ triterpenoids synthesis enzymes $\rightarrow$ triterpenoids; (3) ANE $\rightarrow$ receptor $\rightarrow$ other pathways independent of NO, H_2O_2 and JA $\rightarrow$ TFs $\rightarrow$ triterpenoids synthesis enzymes $\rightarrow$ triterpenoids. In summary, ANE elicitation improved the triterpenoids accumulation in the suspension cultured *C. paliurus* cells via a complex signal transduction network, in which NO, H_2O_2 and JA were involved, and JA was the critical signal molecule.

RNA-seq and de novo assembly

Transcriptome sequencing technology, also known as RNA-seq, is now widely used to explore the mechanism of biological phenomena from the perspective of gene expression difference [36]. In the present paper, three cDNA libraries from the cultured *C. paliurus* cells, named CK (control), 20h (cells elicited for 20 h), 60h (cells elicited for 60 h) respectively, were sequenced using Illumina Hiseq 4000 platform, which generated 65,074,242, 66,016,592 and 53,951,932 raw reads separately (Table S1). After data filtering and stringent quality evaluation, clean reads were obtained. Subsequently, using Trinity program, these clean reads were assembled into 88,144 transcripts with an N50 of 1,590 bp and the average length of 869 bp, which were then joined into 67230 unigenes with an N50 of 1375 bp and the average length of 744 bp (Table S2). Furthermore, the length and number of transcripts and unigenes were statistically analyzed. As shown in Fig.S1, the length of transcripts and unigenes ranged from 201 to 17,098 bp and the majority distributed in 201-400bp, accounting for 45.56% and 52.99% respectively. The above-mentioned data indicated that the generated unigenes in our experiments were of fine quality, and therefore suitable for further annotation.

Function annotations and classifications of unigenes

A total of 33,470 unigenes (accounting for 49.78% of all unigenes) were successfully annotated against seven public databases such as NR, Swiss-Prot, KOG KEGG, GO, COG (Table S3). The results of unigenes homology searches against the NR database were shown in Fig.S2, including the similar, E-value and species distribution. As seen in Fig.S2(c), 3972 annotated unigenes of *C. paliurus* had the first top matches with sequences from *Vitis vinifera*, followed by the *Theobroma cacao* (3417 unigenes), *Prunus persica* (2702 unigenes), *Prunus mume* (2548 unigenes) and *Morus notabilis* (1807 unigenes).

All *C. paliurus* unigenes were aligned to the COG database for prediction and classification by possible function. Overall, a total of 7,694 unigenes (11.44%) were classified into 25 COG functional categories (Fig S3(a)). Our results indicated that 1,008 annotated unigenes (13.10%) fell into “General function prediction only” group, which was the largest among the 25 COG functional categories, followed by “Signal transduction mechanisms” (966, 10.11%). It should be noted that no unigenes was classified as “Extracellular structures” and “Nuclear structure”, which need to be further studied.

According to the annotation against Gene Ontology (GO), altogether 19398 unigenes were classified into three main categories: “cellular component” (42,253 unigenes, 32.91%), “molecular function” (24,703 unigenes, 19.24%), and “biological process” (61440 unigenes, 47.85%), which were further divided into 65 sub-categories. (Fig.S3 (b)). Within the cellular component category, cell sub-category (8,221 unigenes, 19.46%) and cell part sub-category (8,221 unigenes, 19.46%) were the two most significant representation. Among the molecular function unigenes, the majority were classified into “binding sub-category” (11,100 unigenes, 44.93%) and “catalytic activity sub-category” (10,098 unigenes, 40.88%).

To better understand the biological functions of these unigenes in the cultured *C. paliurus* cells, a total of 13,634 unigenes (20.28%) were mapped to 348 KEGG pathways and divided into five branches including Metabolism; Genetic Information Processing, Environmental Information Processing, Environmental Information Processing, Cellular the Processes and Organismal Systems (Fig.S3 (c)). The most

represented pathways were Metabolic pathways (2778 unigenes, 18.74%), followed by biosynthesis of secondary metabolites (1336 unigenes, 9.80%). In addition, 29 metabolic pathways play a significant role in the growth and metabolism of the cultured cells (Table S4), such as phenylpropanoid biosynthesis (179 unigenes), terpenoid backbone biosynthesis (75 unigenes) and flavonoid biosynthesis (42 unigenes). Our annotation against KEGG provided lots of useful information to interpret the metabolic characteristics of the cultured *C. paliurus* cells.

Overall analysis of differentially expressed genes

From the perspective of differentially expressed genes (DEGs), ANE elicitation caused great changes in metabolism related genes expression (Fig. S4), which might further result in markedly changes of the metabolites synthesis and accumulation in the cultured *C. paliurus* cells. A total of 24,788 DEGs were found between the un-elicited cells and elicited cells treated for 20 hours, which consisted of 12,699 up-regulated DEGs and 12,089 down-regulated DEGs. Among these DEGs, 774 genes were significantly up-regulated, while 876 were significantly down-regulated. Similarly, between the un-elicited cells and elicited cells treated for 60 hours, there were 340 significantly up-regulated genes and 129 significantly down-regulated genes. Furthermore, cells elicited for 20 hours showed different gene expression profile by comparison with that elicited for 60 hours. Between these two treated groups, 406 significant up-regulation and 224 significant down-regulation genes were identified. The three groups of DEGs were further analyzed. Among them, 1650, 469 and 630 genes were significantly differentially expressed, and 35 genes belonged to the differentially expressed genes in three samples. The Venn diagram is shown in Fig. S5.

Analysis of DEGs involved in JA synthesis pathway under ANE treatment

To further validate the key role of JA in the responses of ANE elicitation in *C. paliurus* cells, the expression levels of JA synthesis-related genes such as *LOX*, *AOS*, *AOC*, and *OPR* were detected using comparative transcriptome sequencing. A total of 7 annotated candidate *LOX* unigenes were up-regulated, among which 1 were significantly up-regulated (Table 1). Within the 6 up-regulated *OPR* unigenes, 2 were found to be significantly up-regulated. Meanwhile, the expression of 2 *AOC* and 1 *AOS* unigenes increased, but not significantly (Table 1). The above DEGs analysis associated with JA synthesis was in consistence with the determination data of JA concentration in the elicited cells. Our results indicated that ANE elicitation activated the expression of JA synthesis pathway genes, which finally enhanced the triterpenoids accumulation in the culture cells.

Analysis of DEGs involved in JA signal transduction pathway under ANE treatment

Researches showed that JAZ and JAR1 were key transcription factors in JA signal transduction pathway, and played critical roles in the synthesis of secondary metabolites [37, 38]. In the present paper, a total of 7 JAZ candidate unigenes were annotated, among which 5 were found to be down-regulated, and 1 kept almost unchanged, while 1 was up-regulated (Table 2). In addition, one JAR1 candidate unigene (c39500_g1) was annotated and observed to be up-regulated, whose FPKM increased from 48.614 to

77.295 at the 20th hours after ANE elicitation (Table 2). Consequently, it was speculated that the down-regulation of JAZs together with the up-regulation of JAR1 mediated the ANE elicitation signal transduction, and led to the rising of triterpenoids synthesis in the cultured cells, which further validated the above deduced JA signal pathway and the critical role of JA in ANE elicitation.

Discussion

Fungal elicitor, as a highly effective stimulator, can trigger rapid defense responses when applied to in vitro plant cell, tissue and organ cultures, and thus stimulate the secondary metabolites synthesis by activating the expression of specific genes [39, 40]. Our elicitation experiment suggested that ANE can significantly improve the triterpenoids yield of the cultured *C. paliurus* cells, and therefore has a promising application prospect in the production of plant-derived triterpenoids. However, more investigations are still needed to better understand the elicitation mechanism. Our previous report showed that ANE elicitation up-regulated the expression of triterpenoid synthesis pathway genes, and therefore enhanced the triterpenoid accumulation in the cultured *C. paliurus* cells [41], but the involved signal mechanism causing the triterpenoid synthesis genes up-regulation hasn't been revealed yet.

Researches indicated that there are four common signal molecules, including NO, H₂O₂, JA and SA, which mediate signal transduction when plants face various environmental stresses, such as fungal invasion, insect attack, drought, osmotic pressure [42–44]. Usually, these signal molecules play their roles in the complicated signal transduction network harmoniously, and result in differential expression of some related key genes, which further causes various physiological responses in the plants [45, 46].

Signal transduction is a very complex biological process, and therefore has been attracting many researchers' attention in recent decades. Domingos et al. [47] comprehensively reviewed the characteristics, signal transduction pathways and mechanisms, and functions of NO as a plant signal molecules, and pointed out that NO played an important role in responses of plants facing various kinds of biotic and abiotic stress. Turner et al. [48] gave a detailed elaboration on JA signal pathway, as well as JA's biosynthesis and regulation, signaling functions, and relationship with other signal molecules like SA and ethylene. Although lots of related researches have been well carried out, signal transduction mechanisms such as signal molecules crosstalk have not been fully understood yet, because numerous highly complicated pathways are involved as well as various signal molecules and related proteins. What's more, there are too many plant species in the world, and different plant may have different transduction mechanisms.

The research of Xu and Dong [49] suggested that nitric oxide mediated the fungal elicitor-induced taxol biosynthesis in the *T. chinensis* suspension cultured cells through both reactive oxygen species-dependent and -independent signal pathways. As shown in their report, both NO quenching and reactive oxygen species (ROS, including H₂O₂) blocking inhibited the elicitor-induced taxol production, which was in accordance with the results of our experiment performed in the suspension cultured *C. paliurus* cells. Their data also showed that NO quenching suppressed the H₂O₂ synthesis, and exogenous NO addition

increased the H₂O₂ accumulation, however according to our results, the change of NO concentration had no effect on the H₂O₂ synthesis. According to the schematic representation of action mode of elicitor in plant cell given by Halder et al. [20], NO synthesis was the downstream event of the ROS burst under elicitation, which coincided with our conclusion. These different phenomena and results may be caused by plant species differences or other unknown reasons, and further studies are still needed.

Both SA and JA are important signal molecules associated with plant defense responses [50]. Our previous studies showed that any one of SA and JA inducing could stimulate the synthesis of secondary metabolites [51]. However, according to Li et al. [52], there was antagonism between SA and JA pathways in *A. thaliana* in response to elicitation, and WRKY70 acted as an activator of SA-induced genes and a repressor of JA-responsive genes to integrate signals from these mutually antagonistic pathways. Similar results were reported by Xu et al. [53]. In our ANE elicitation experiments, JA concentration increased significantly after the ANE addition, and played a critical role in stimulating triterpenoids synthesis, while SA concentration was lower than that of the untreated cells. Our results also confirmed the antagonism between SA and JA pathways.

In this study, JA was found to be not only the junction of NO and H₂O₂ signal pathways but also the critical point in the whole signal network of ANE elicitation. Researches indicated that JA, as an important endogenous phytohormone, was synthesized in plant using α -linolenic acid as starting compound [54], and a series of cascade enzymes of its synthesis pathways were involved, including *LOX*, *AOS*, *AOC*, and *OPR* [55, 56], which were also identified in our elicited *C. paliurus* cells (Table 1). Our RNA-seq analysis showed that a total of 3 candidate JA synthesis pathway genes including 1 *LOX* and 2 *OPR* were found to be significantly up-regulated under the ANE stimulation,, as well as 5 down-regulated JAZs and 1 up-regulated JAR1 relating to JA signal transduction, which further validated the key role of JA in response to ANE elicitation. JAs (jasmonates) usually refer to a category of organic compounds derived from JA, including jasmonic acid (JA), methyl jasmonate (MeJA), and jasmonic acid isoleucine (JA-Ile), which acted as ubiquitous and conserved regulators for the production of secondary metabolites in the most majority of plants, from gymnosperms to angiosperms [37, 57]. It is generally recognized that JA shows its effects through a bioactive form - JA-Ile, which is synthesized from JA and isoleucine under the catalysis of JA amino acid synthetase (JAR1) [37, 38]. Normally, JA-Ile concentration is very low, and MYC2 is bound by jasmonate ZIM-domain protein (JAZ) and presents in an inactive form; when the JA-Ile concentration increases to a certain level under stimulation, the COI1 protein of SCF^{COI1} complex interacts physically with JAZ protein and make the latter to be labeled with ubiquitin, which causes MYC2 to be freed from JAZ and JAZ to be degraded by 26S-proteasome; the free MYC2 released from JAZ activates the expression of the early jasmonate-response genes and finally improved the synthesis of secondary metabolites to adapt to the environment changes [37, 57–60]. Our results also indicated that JA mediated the signal transduction of ANE elicitation, in which TFs like JAZ and JAR played important roles. Meanwhile, MYC2 were also annotated in our ANE treated cells, but its expression declined slightly at the 20th hour after elicitation (Table 2). However, further studies are still needed to interpret the underlying mechanism of these TFs associated with JA signal transduction pathways.

Conclusion

ANE elicitation significantly enhanced the triterpenoids accumulation in the suspension cultured *C. paliurus* cells. NO, H₂O₂ and JA were all involved in this elicitation as important signal molecules. Three related signal pathways were deduced based on a series of experiments of signal molecules blocking and exogenous addition, together with three hypothetical signal transduction pathways involved in ANE elicitation. JA was not only the junction of NO and H₂O₂ signal pathways but also the critical point in the whole signal network of ANE elicitation. RNA-seq analysis further validated the pivotal role of JA in ANE elicitation. As far as we know, up to now this paper is the first report on the signal molecules crosstalk involved in the ANE elicitation in the suspension cultured *C. paliurus* cells.

Declarations

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Authors' contributions

ZY and JC conceived the idea and designed the experiments. MW and QX performed the experiments. MW, XX and XZ contributed to data analysis. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All relevant data are within the paper and its Supporting Information files.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1
Summary of differentially expressed genes in JA synthesis pathway of suspension cultured *C. paliurus* cells

Unigene name	Unigene code	FPKM of CK	FPKM of E _{20h}	Trends
LOX	c41476_g2	32.342	36.76	up
	c33483_g1	16.031	16.534	up
	c41476_g3	74.344	329.943	up*
	c17385_g1	22.275	25.741	up
	c33483_g1	16.031	16.534	up
	C45031_g1	17.787	22.979	up
	c17385_g1	22.275	25.741	up
AOS	c43559_g1	0.618	2.196	up
AOC	c10965_g1	38.972	41.961	up
	c35833_g1	58.4	68.947	up
OPR	c24740_g1	8.136	16.473	up
	c24761_g1	3.648	4.199	up
	c32622_g1	19.969	163.079	up*
	c36002_g1	648.51	853.441	up
	c36002_g2	519.983	1052.36	up
	c39431_g1	160.52	584.483	up*
Note: LOX: lipoxygenase; AOS: allene oxide synthase; AOC: allene oxide cyclase; OPR: oxo-phytodienoic acid reductase.				
CK: the control group cells; E _{20h} : The cultured cells elicited by ANE for 20 hours.				
Superscript symbol “*” indicates that this unigene expression changed significantly.				

Table 2

Summary of differentially expressed genes in JA signal transduction pathway of suspension cultured *C. paliurus* cells

Unigene name	Unigene code	FPKM of CK	FPKM of E _{20h}	Trends
JAR1	c39500_g1	48.614	77.295	up
JAZ	c27861_g1	10.327	4.938	down
	c33284_g1	2.664	5.504	up
	c38204_g1	9.757	8.196	down
	c44879_g3	24.427	24.881	up
	c44879_g4	58.892	32.683	down
	c45306_g2	38.711	31.479	down
	c45306_g3	44.28	26.045	down
MYC2	c29186_g1	27.69	21.089	down
	c37338_g1	16.89	14.904	down
	c43784_g1	103.877	85.936	down
Note: JAR1: JA-amino acid synthetase 1; JAZ: jasmonate ZIM domain-containing protein; MYC2: myelocytomatosis protein 2.				
CK: the control group cells; E _{20h} : The cultured cells elicited by ANE for 20 hours.				

Figures

Fig.1

Figure 1

Comparison on biomass (a) and triterpenoids yield (b) between the ANE elicited and un-elicited *C. paliurus* cells. Suspension culture conditions: *C. paliurus* cells were cultured in MS medium supplemented with an combined hormones (1.0 mg/L KT+0.5 mg/L 2, 4-D+0.3 mg/L NAA), 6 g/L agar and 30 g/L sucrose, and cultivated in the dark at $25 \pm 1^\circ\text{C}$ on a rotary shaker at 115 rpm. ANE elicitation parameters: ANE at a concentration of 200 $\mu\text{g/mL}$ was added into the culture medium on the 4th day after cell inoculation; cells were respectively harvested at 0th, 20th, 40th, 60th and 80th hours after the elicitation of ANE. CK: the control group cells; ANE: The cultured cells elicited by ANE.

Fig.2

Figure 2

The change profiles of NO (a), H₂O₂ (b), JA(c) and SA (d) concentration of the suspension cultured *C. paliurus* cells at different time after ANE elicitation. Suspension culture conditions: *C. paliurus* cells were cultured in MS medium supplemented with an combined hormones (1.0 mg/L KT + 0.5 mg/L 2, 4-D + 0.3 mg/L NAA), 6 g/L agar and 30 g/L sucrose, and cultivated in the dark at 25 ± 1 °C on a rotary shaker at 115 rpm. ANE (200 μg/mL) was added into the culture medium on the 4th day after cell inoculation. CK: the un-elicited cells; ANE: The cultured cells elicited by ANE.

Fig.3 **Figure 3**

Change profiles of NO, H₂O₂ and JA contents, biomass, and triterpenoids yield of the cultured *C. paliurus* cells causing by NO quenching and exogenous addition. a: the contents of NO in cultured *C. paliurus* cells under different concentrations of C-PTIO; b: biomass of the cultured *C. paliurus* cells under different concentrations of C-PTIO; c: the contents of H₂O₂ in the ANE elicited *C. paliurus* cells with or without the addition of the 100 $\mu\text{mol/L}$ C-PTIO; d: the contents of JA in the ANE elicited *C. paliurus* cells with or

without the addition of the 100 $\mu\text{mol/L}$ C-PTIO; e: the yield of triterpenoids in the ANE elicited *C. paliurus* cells with or without the addition of the 100 $\mu\text{mol/L}$ C-PTIO; f: the contents of H_2O_2 in the ANE or SNP (adding 150 $\mu\text{mol/L}$ SNP, exogenous NO donor) elicited *C. paliurus* cells; g: the contents of JA in the ANE or SNP elicited *C. paliurus* cells; h: the yield of triterpenoids in the ANE, ANE combined with 100 $\mu\text{mol/L}$ C-PTIO, and SNP treated *C. paliurus* cells CK: the control group cells; ANE: the cultured cells elicited by ANE; ANE+ C-PTIO: the cultured cells adding ANE and C-PTIO; SNP: the cultured cells adding to SNP; ANE+ C-PTIO +SNP: the cultured cells adding to ANE, C-PTIO and SNP.

Fig.4

Figure 4

Change profiles of H2O2 and JA contents, biomass, and triterpenoids yield of the cultured *C. paliurus* cells causing by H2O2 blocking and exogenous addition. a: the contents of H2O2 in cultured *C. paliurus* cells under different concentrations of DMTU; b: biomass of the cultured *C. paliurus* cells under different concentrations of DMTU; c: the contents of JA in the ANE elicited *C. paliurus* cells with or without the addition of the 2 mmol/L DMTU; d: the yield of triterpenoids in the ANE elicited *C. paliurus* cells with or without the addition of the 2 mmol/L DMTU; e: the contents of JA in the ANE, ANE combined with 2 mmol/L DMTU and 50 μ mol/L H2O2 elicited *C. paliurus* cells; f: the yield of triterpenoids in the ANE, ANE combined with 2 mmol/L DMTU, and 50 μ mol/L H2O2 treated *C. paliurus* cells CK: the control group cells; ANE: the cultured cells elicited by ANE; ANE+ DMTU: the cultured cells adding ANE and DMTU; H2O2: the cultured cells adding H2O2; ANE+DMTU+ H2O2: the cultured cells adding ANE and DMTU.

Fig.5

Figure 5

Change profiles of JA contents, biomass, and triterpenoids yield of the cultured *C. paliurus* cells causing by JA inhibiting and exogenous addition. a: the contents of JA in cultured *C. paliurus* cells under different concentrations of NDGA+IBU; b: biomass of the cultured *C. paliurus* cells under different concentrations of NDGA+IBU; c: the yield of triterpenoids in the ANE elicited *C. paliurus* cells with or without the addition of the 100 $\mu\text{mol/L}$ NDGA+IBU; d: the yield of triterpenoids in the ANE, ANE combined with 100 $\mu\text{mol/L}$ NDGA+IBU, and MJA(adding10 $\mu\text{mol/L}$ MJA , exogenous JA donor) treated *C. paliurus* cells. CK: the control group cells; ANE: the cultured cells elicited by ANE; ANE+ NDJA+IBU: the cultured cells adding ANE and NDJA+IBU; ANE+ NDJA+IBU +MJA: the cultured cells adding ANE, NDJA+IBU and MJA.

Figure 6

The deduced signal transduction network of ANE elicitation in the suspension cultured *C. paliurus* cells. Blue arrow line (including solid line and dotted line): signal transduction pathway deduced according to the resultant analyses in the present paper; purple dotted arrow line: hypothetical signal transduction pathway.

Supplementary Files

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