Identification of novel anthocyanins synthesis pathway from fungus, Aspergillus sydowii H-1

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Abstract

Background Anthocyanins are common substance in people's production and life. Usually it was used as a natural dye, more and more studies have shown that anthocyanins have potential preventive and / or therapeutic effects on human health, such as improving cardiovascular function and treating obesity. However, anthocyanins are generally thought to occur only in natural plants. The difficulty of mass production limits the application of anthocyanins in industry. Production of anthocyanins from microbial fermentation is not restricted by time, space and environmental conditions as compared with plants, which makes it more anxious to obtain anthocyanins from microorganisms. Here, we firstly reported a fungus, Aspergillus sydowii H-1which can produce anthocyanins under the specific fermentation, and analyzed its metabolome and transcriptome. Results All of the 31 gene loci related to anthocyanins synthesis pathway were identified in A. sydowii H-1, and almost all of those genes display high correlation with the data from metabolome. Among them, chalcone synthase gene (CHS) and cinnamate-4-hydroxylase gene (C4H) were only found using denovo assembly without reference, and interestingly, the best hits of these two genes all belong to plant species. Therefore, the plant-derived genes, CHS and C4H, may be one of the reasons why A. sydowii H-1 can produce anthocyanins. We also identified 530 lncRNAs in our datasets, and among them, three lncRNAs targeted to the genes related to anthocyanins biosynthesis via cis-regulation, which likewise provide clues for the underlying mechanism of anthocyanins' production in the fungi. Conclusions In this study, we discovered a fungus, Aspergillus sydowii H-1, which can produce anthocyanins under the specific fermentation condition. It's the first report that anthocyanins can be produced in non-plant species.

Background

Natural pigments are generally extracted from plants (carrots, radishes and grapes, etc.), insects (*Dactylopius coccus*), fungi (monascus) even cyanobacteria (*Arthrospira spp*). The well-known natural pigments are composed of carotenoids, benzopyran derivative pigments (anthocyanins, flavonoid pigments), quinoid pigments (benzoquinone and anthraquinone), tetrapyrrole derivatives (chlorophyll and heme), N-heterocyclic compounds (purine and flavone) and melanin .They have a wide range of application due to their excellent biological activities such as antibacterial, anticancer, anti-oxidation, hypolipidemic function and so on [1].

Among the most popular natural pigments, anthocyanins is water-soluble and a class of flavonoids [2]. At present, there are six common anthocyanins: pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp). Anthocyanins are naturally glycosylated, and the most common sugar ligands are hexose (glucose and galactose) and pentose (arabinose, rhamnose and xylose) [3]. Currently, anthocyanins have gained increasing attraction based on their color diversity and nutritional characteristics. The commercial demand for pigments to color foods and cloths made anthocyanins were widely used in food and textile industries [4]. For its health benefits including antioxidant activity, anti-aging activity, bacteriostatic activity, the ability to reduce the risk of cancer and diabetes, and the role of cardiovascular health protection, anthocyanins were also applied in skin care and health care products

[5]. Additionally, anthocyanins with the O-diphenol structure has the ability to inhibit tumor growth and metastasis which may be applied in pharmaceutical industries [6]. In summary, the extensive application value of anthocyanins makes the study of anthocyanins synthesis become the focus of attention in the world.

In plants, biological synthesis pathway of anthocyanins has been researched detailly. Firstly, phenylalanine was converted into 4-coumaryl CoA which is regulated by phenylalanine lyase (PAL), cinnamate hydroxylase (C4H) and 4-coumaroyl CoA ligase (4CL); Secondly, dihydroflavonol was derived from 4-coumaryl CoA with the help of chalcone synthase (CHS), chalcone isomerase (CHI) and Flavanone -3- hydroxylase (F3H). Then dihydroflavonol was transformed into anthocyanins with the help of dihydroflavonol reductase (DFR), flavonoid 3'- hydroxylase (F3'H) and leucoanthocyanidin dioxygenase (LDOX). After that, the glycosylation of anthocyanins is regulated by flavonoid glycosyltransferase (UGTs) [7, 8]. Finally, Pg and Mv were synthesized from Cy and Dp separately with help of O-methyltransferase (OMT).

Once a kind of anthocyanins was identified, an important limiting factor for its discovery is the ability to produce qualities of this chemical compound. As known, plant-derived anthocyanins are neither stable nor sustainable, because plant growth fluctuates with seasonal and environmental conditions [9]. Due to the high production and no restricts in the space and other conditions, microorganisms were thought as a source to gain mass production of anthocyanins. Generally, microorganisms can not only save the production cost, but also increase the pigment yield [10, 11]. So far, many compounds that we used to thought they were plant-derived have been founded in microorganisms, such as betalain in *penicillium novae-zelandiae* [12], lawsone, an orange-red pigment in *gibberella moniliformis* [13], taxol and related taxanesin *Aspergillus niger* [14] etc. However, no clear studies shown that anthocyanins can be produced from microorganisms directly. But it was reported that the enzyme activities of PAL, C4H and 4CL were detected during *Alternaria sp.* MG1 fermentation [15]. These results suggest that microorganisms may have the potential to synthesize anthocyanins directly.

A. sydowii was often reported as a pathogen of gorgonian corals [16] and capable of growing in the sea [17]. But it is also founded in different habitats where it survives as a soil decomposing saprotroph [18, 19]. It was studied widely for its biodegradation of agrochemicals and contaminants including polycyclic aromatic hydrocarbons [20], trichlorfon [21], methyl parathion [22], xenobiotics [20] and so on. But some novel secondary metabolites, anti-diabetic and anti-inflammatory sesquiterpenoids [23], 2-hydroxy-6-formyl-vertixanthone and 12-O-acetyl-sydowinin A [24], sesquiterpene and xanthone [25], indole alkaloids [26] etc., were identified from *A. sydowii* which indicated that the complexity and capability of fungal genes in orchestrating the biosynthetic routes of their secondary metabolites. These compounds are produced to protect the fungal from external coercion. For better surviving, it can synthesis cellulase [27] and xylanase [28] to degrade of lignocellulose synergistically.

Long non-coding RNA (lncRNA) is a kind of RNA which was defined as longer than 200nt and without the capacities of coding protein or with very low ability [29]. LncRNA existed in plants extensively and

involved in the process in regulating plant growth and development, stress response and so on [30]. For example, TPSI1/Mt4 gene family was studied thoroughly in responding to phosphorus stress in tomato, rice, *Arabidopsis thaliana* [31]. Another study showed that exceed 300 kinds of lncRNAs were found in *Arabidopsis thaliana* under various stress stimuli (cold, drought, salt, heat and strong light, etc.) [32]. Hence, lncRNA was considered as regulating components to help plants cope with stress [33].

We have isolated and purified a fungus from humus which can produce a kind of water-soluble purple pigments, which was named *A. sydowii* H-1. This study confirmed that the *A. sydowii* H-1 purple component are anthocyanins via FTIR and metabolomics methodologies and explored the anthocyanins synthesis pathway of *A. sydowii* H-1 by transcriptome. The results of this study firstly provide new sources of anthocyanins, which may provide new strategies and perspectives for the production of anthocyanins.

Results

Preliminary identification of anthocyanins and the related properties of fermentation

With the increase of fermentation time, the concentration of pigments gradually deepened (Fig.1A). The scanning result (Fig.1B) showed that the fermentation broth has an absorption peak at 520nmwhich is consistent with the absorption peak of anthocyanins [34]. And the FTIR spectra result of freeze-dried powder of purple fermentation liquid of *A. sydowii* H-1 was showed in Fig.1C. The stretching band at 3394.10 cm^{-1} corresponds to OH vibration of hydroxyl group [35]. The major peaks observed for chitosan were 1647.07 cm^{-1} (amide I band) [36], peaks around 800–1150 cm^{-1} are characteristic of polysaccharides, assigned to C–O valence vibrations and C–O–C stretching vibrations of carbohydrates, including fructose, glucose and glucomannan. Peaks between 1133 and 1457 cm^{-1} correspond to phenolic compounds such as anthocyanins and condensed tannins [37].

While *A. sydowii* H-1 is in a growth delay period of 1-2 days, glucose consumption is slow and there has no pigments were produced. During 3-7 days, the fungi are in logarithmic growth phase and the mycelium grows rapidly with rapidly glucose consumption. The UV-visible spectrum showed that the characteristic absorption at 520 nm peak of the purple pigments is gradually increased indicating the accumulating of purple pigment. At 8th day, the fermentation broth reached the maximum pigment absorbance, and the fungi were in stable phase with slow growth. The weight of the cells decreased in the last three days, and the hyphae began to disintegrate because of self-melting. At the same time, the mycelium has no longer produced purple pigments (Fig.1D).

In this study, the growth of *A. sydowii* H-1 purple pigment was basically consistent with the consumption of glucose. According to the growth curve of *A. sydowii* H-1 and the production of pigments, the two key points of *A. sydowii* H-1 fermentation are 2th day, the pre-growth period with no significant accumulation of pigments, and 8th day, the stable period of the cells with the highest accumulation of pigments. So, we

selected the fermentation broth and cells on the second (G2) and eighth (G8) days for subsequent metabolome and transcriptome analysis.

Widely targeted flavonoids metabolomics

Comparing the metabolites in G2 with G8, the mean value of five anthocyanins, peonidin o-malonylhexoside (Peonidin-Mh), cyanidin 3-O-glucoside (Kuromanin), cyanidin, malvidin 3-O-galactoside (Malvidin-3Ga), malvidin 3-O-glucoside (Oenin) in G2 all are 1.21E-06 calculated by forced integral, which is not reach the detection limit, indicating that anthocyanins were not detected based on UPLC-QTRAP-MS/MS technology. On the contrary, the five anthocyanins contents significantly increased in G8 and the mean value is 2.19E-03, 3.18E-04, 1.56E-03, 7.42E-03 and 9.98E-03, separately (Table.S2). These kinds of anthocyanins have been reported in berries (*Lonicera caerulea*, *Rubus fruticosus, Ribes nigrum* and *Morus alba*), cereals (*Zea mays*) and vegetables (*Brassica oleracea*, *Dioscorea alata, Daucus carota* and *Asparagus officinalis*) [38-40]. The above studies showed that the purple pigments in *A. sydowii* H-1 are anthocyanins.

Transcriptome sequencing, transcripts construction and the analysis of differentially expressed genes

RNA-Seq was used to construct the transcripts of *A. sydowii* H-1 in both the second (G2) and eighth (G8) day with three biological replicates, respectively. After the removal of adaptor-contaminated, low-quality and rRNA reads, the clean reads from RNA-seq were aligned to *Aspergillus sydowii* CBS 593.65 [41] by Hisat2 2.0.4 [42], and the mapped ratio ranges from 78.47 to 91.04%. The mean of GC percentage and Q30 percentage are 53.41% and 94.16% (Table. 1), respectively. A high Q30 value indicates that the sequencing data is authentic. Reference-guide and denovo assemble method were used to obtain transcripts as complete as possible (see method). Totally, 13,045 gene loci are consisting of 15,161 transcripts including 14,376 reference-guide derived transcripts and 785 denovo derived transcripts. The expression levels of transcripts were calculated with RSEM [43] software. Pearson correlation analysis was performed on the expression matrices of genes (Fig.S1). The sample correlation analysis showed that there was a difference trend between T5 and T4, T6 in the same repeat, which may due to the different growth of fungi in the same fermentation stage, so the subsequent expression related analysis will not include T5. Gene differential expression analysis identified 5,243 differentially expressed genes (DEGs) ($|\text{fold change}| \geq 2$ and P-value ≤ 0.05) (Table S3).

Identification of anthocyanins related genes

Although the synthesis pathway of anthocyanins in plants has been studied in detail, the anthocyanins-related genes in fungi have not been fully reported yet. Finally, total of 31 anthocyanins-related genes were identified (Table 2, Fig2.A). According to Pelletier's study [44], we divided these genes into early

biosynthetic genes (EBGs) and late biosynthetic genes (LBGs). Among them, *4CL* is the key to the general phenylpropanoid pathway and participates in monolignol biosynthesis through the production of p-coumaroyl-CoA [45] which has the largest number of homologous genes. It's worth noting that *C4H* and *CHS* are only founded in our own denovo assembled unigenes. Cinnamate 4-Hydroxylase (C4H, EC 1.14.13.11) is the second enzyme of phenylpropanoid pathway and a member of cytochrome P450 family. Chalcone synthase (CHS, EC 2.3.1.74) is a key enzyme that catalysis the first committed step in the flavonoid biosynthetic pathway. Moreover, the best Blast hits of these two genes are in plant species with very high identity and query coverage, although those genes have been reported in other fungi (Table. S4). So, we guess that the one of the major reasons why the *A. sydowii* H-1 can produce anthocyanins because these two plant-derived genes are transferred into fungi by some ways. All of anthocyanins related genes are random selected for RT-qPCR to verify the accuracy of RNA-seq (Fig.S2).

Except *CHS* and *C4H*, all of other genes have been found the best blast hits in other fungi species. In order to explore the evolutionary relationship of remained anthocyanins related genes between fungi and plants. Leucocyanidin oxygenase gene (*LDOX*), flavanol synthase gene (*FLS*) and flavanone 3-dioxygenase gene (*F3H*) in *A. sydowii* H-1 which all belong to the 2-oxoglutarate-dependent oxygenases (2-ODD) family were used to construct phylogenetic tree with some known homologous sequences in other fungi and plants (Fig.3). The results showed that all of these three types of genes are clearly separated according to fungi or plant rather than the type of genes. This suggests that the anthocyanins synthesis pathway genes evolved separately in plants and fungi. Furthermore, these genes in plants can be clearly divided into different branches according to different gene types. However, in fungi, these genes are difficult to separate clearly. This manifestation may indicate that these three types of genes in plants are differentiated earlier than in fungi.

Analysis of lncRNA genes related to anthocyanins

LncRNAs performed a variety of functions in different important biological processes [46]. However, the regulation of lncRNA on anthocyanins synthesis has not been adequately reported. After filtering, total of 530 lncRNAs were identified in *A. sydowii*H-1, 146 of them showed significantly differential expression (|fold change|≥2, P-value≤0.05). Among them, 144 lncRNAs regulated target genes by either cis-acting or trans-acting (Fig3.A). We followed the two steps below to select lncRNAs which may interact with anthocyanins synthesis related genes: 1) The target genes of lncRNA must be one of the anthocyanins synthesis related genes (|cor| ≥ 0.7, P-value ≤ 0.05). 2) There should be a correlation between lncRNAs and at least one kind of anthocyanins compounds (|cor|≥0.7, P-value≤0.05). Finally, a total of three lncRNAs regulate two genes involved in anthocyanins synthesis. One functional gene, ASPSYDRAFT_91437, three lncRNAs genes, MSTRG.7153.1, MSTRG.3144.1, and MSTRG.3145.1 were differentially expressed in the G2 and G8, respectively (Fig4.B, Table3).

The correlation between genes, including functional genes and lncRNAs genes involved in anthocyanins synthesis, and major flavonoids compounds is presented as a heatmap (Fig4.C). Interestingly, the highest

and most significant positive correlation was found between 4cl and flavonoids compounds. Which suggested that 4cl may acts as a gatekeeper in the flavonoid biosynthesis pathway and plays an important role in regulating the biosynthesis of anthocyanins.in *A. sydowii* H-1

Discussion

We firstly identified anthocyanins in *A. sydowii* H-1 which is a new organic source for its production and reported the synthesis of anthocyanins in microorganism naturally. Microbial-derived anthocyanins are more suitable for industry because of they are easier obtained on a large scale production and extracted without expensive and harmful organic solvents [47]. The previous research suggested the production of plant-derived anthocyanins is limited to the plant species and the environment where the plants locate [48]. This study found that *A. sydowii* H-1 can directly produce anthocyanins, which has significance to the industrial production of anthocyanins.

Although merged two assembling methods to construct the final transcripts in *A. sydowii* H-1, we find many cytochrome p450 reductase gene (*CPR*) in our data pool but still not find definite flavonoid 3'-hydroxylase gene (*F3'H*) which belongs to *CPR* gene family, so the specific *F3'H* needs to be further identified.

RNA transcription is a tightly regulated process. Non-coding RNAs can target multiple aspects of the process, including targeting transcriptional activators or transcriptional repressors, components of transcriptional reactions such as RNA polymerase (RNAP) II, and even DNA double helix structures to achieve the purpose of regulating gene transcription and expression [49]. Although most studies have identified lncRNAs in anthocyanins-producing plants, among which only Yuanxiu Lin et al. found that lncRNAs in strawberry may regulate anthocyanins synthesis by competitively binding miRNA [50]. The others have not linked lncRNAs to target genes involved in the anthocyanins synthesis pathway [51-53]. We predicted three lncRNAs involved in the regulation of anthocyanins-related genes, as one of the other possible mechanisms for the production of anthocyanins in fungi, which providing a possible direction for exploring the mechanism of anthocyanins production.

Conclusion

We isolated a fungus, *Aspergillus sydowii* H-1, that produces anthocyanins by fermentation. The genes related to anthocyanins synthesis were identified by transcriptome sequencing and metabolome analysis, as well as three lncRNAs involved in the regulation of anthocyanins-related genes.

Methods

Extracting and purifying the purple pigments of *Aspergillus sydowii* H-1

Aspergillus sydowii H-1 was isolated from the humus in Chengdu, China and cultured on Czapek Dox agar medium. Spore suspension was prepared from 7-day-old culture slants by adding adequate amount of sterile distilled water. The spore number was 1.6×106 cells/mL and inoculated into 200 mL seed culture (Chest's medium) at 28℃ 180 rpm/min for 60 h. Then 10mL of the above mycelial suspension (5% v/v) was inoculated into 200 mL of fermentation medium. The 1L fermentation medium composed of 5 g glucose, 3 g peptone, 0.5 g yeast extract, 1 g KH_2PO_4, and 1 g NaCl. The fermentation broth of the 8th day (G8) was collected and purified by DM130 macroporous resin. The elution flow rate is 1.5 mL/min, 70% ethanol at the flow rate of 1 mL/min. Then the G8 fermentation broth treated with DM130 macroporous resin was freeze-dried into powder and stored at 4℃.

Identifying the purple pigments and determining the biochemical properties during fermentation

Three biochemical properties (fungal biomass, pigments yield and the content of glucose) were monitored at first day's interval up to the 11th day, and all experiments were repeated three times. The liquid culture containing pigments was separated from mycelia through water soluble filter of 0.45 μm pore size (Jing Teng, China). Quantitative analysis of glucose used the Dynitrosalicylic Acid (DNS) method. And the biomass concentration was determined by gravimetric method.

Scanning the characteristic absorption peak of purple fermentation broth at 400~800 nm by spectrophotometer (Thermo, U.S.A) and the pigments yield was measured at peak absorption.

Identification of the purple substance by Fourier transform infrared Spectroscopy (FTIR). FTIR spectra determination was acquired using Nexus 6700 (Thermo, USA). The above G8 purple lyophilized powder was thoroughly mixed with KBr and pelletized. The resolution of the obtained spectrum is 0.09cm^{-1}, and the range is 4000-400cm^{-1}.

metabolome analysis of *Aspergillus sydowii* H-1 fermentation broth

Follow the above methods, the lyophilized powder of the second day (G2) and the 8th day (G8) was prepared to downstream analysis. Then, 0.1 g powder in G2 and G8 were extracted overnight with 70% methanol aqueous solution of 1.0mL at 4 ℃ and centrifuged at 10000 rpm/min for 10 min, the supernatant was filtered by 0.22 μm filtration membrane and stored in brown bottle at 4℃ for further metabolome determination.

Chromatography used an Acquity UPLC system (Waters, USA) with a HSS-T3-C18 column (1.7 μm,2.1mm × 100 mm, Waters) and the column temperature was 40℃. The mobile phase consisted of 0.04% (v/v) acetic acid in ultra-pure water (A) and 0.04% (v/v) acetic acid in acetonitrile (B), the initial volume ratio

was set as follows: 5% B at 11.0 min, 95% B at 12.0 min, 5% B at 12.1 min, 5%B at 15min. Mobile phase velocity was 0.4 mL/min and sample injection volume were 5 μL.

The API 6500 QTRAP-MS/MS system equipped the electrospray ionization (ESI) for MS/MS analysis. The obtained data were processed by the software Analyst 1.6.3 (AB SCIEX). Qualitative analysis of metabolites according to the MWDB (metware database) and the metabolite information public database, some of these metabolites are characterized, after removing the isotope signal the repeated signals of K^+, Na^+, NH_4^+, and the repetitive signals of fragment ions of other larger molecular weight substances. Metabolite structure analysis consulted the existing mass spectrometry public databases such as, MassBank (http://www.massbank.jp/), MoToDB (http://www.ab.wur.nl/moto/), KNAPSAcK (http://kanaya.naist.jp/KNApSAcK/), METLIN (http://metlin.scripps.edu/index.php). Metabolite quantification was performed with multiple reaction monitoring (MRM)

RNA extraction, sequencing and analysis

RNA was isolated and cDNA libraries were constructed at the second fermentation day and the eighth fermentation day (three replicates of each time point) according to the Illumina HiSeq X-ten (Illumina, San Diego, CA) RNA library protocol. Library sequencing was done on a HiSeq X-ten (Illumina) platform to obtain 150 bp of paired-end reads. The raw sequencing reads were submitted to National Center for Biotechnology Information (NCBI) (BioProject: PRJNA542911).

Low quality reads were trimmed by Trimmomatic (Version 0.32) [54]. Searching the Rfam database via bowtie2 2.3.2 [55],the rest of reads which can map to the known transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) were removed. Then the trimmed and rRNA-free reads were mapped to *Aspergillus sydowii* CBS 593.65 [41] by Hisate2 (Version 2.1.0) [42], and transcriptome was assembled by StringTie (Version 1.3.3b) [56] by reference-guided method with default parameters. In order to find sequences unique to reference genome, Trinity version 2.8.4 [57] was used to assemble transcripts by denovo method with the default parameters. Denovo assembled transcripts shorter than 300 bp were discarded and the longest transcript in each cluster (unigene) was selected as the representative of the unigene. Comparing reference-guided sequences with denovo nucleotide unigenes by blastn software, the sequences which can align to reference-guided sequences are removed. Remained unigenes were used as the denovo assembly result. Merging the two method assembled sequences were used as the final genes in *A. sydowii* H-1. And the expression levels of genes were calculated and normalized via Expectation Maximization in RSEM [43]. In order to obtain the annotation information of genes, the coding sequence (CDS) and the translated protein sequences of unigenes were predicted with TransDecoder version r20140704 (http://transdecoder.github.io/, accessed 26 Sept. 2018). Then proteins were functionally annotated by blastp (Camacho et al., 2009) based on queries of functional databases, including the SwissProt database and NCBI nonredundant database and Refseq database, Pathway Annotation of the Kyoto Encyclopedia of Genes and Genomes (KEGG) terms were annotated using KOBAS version 3.0 [58],

protein domains were annotated using IterProScan5 version 2.0 (https://github.com/ebi-pf-team/interproscan) against with pfam database.

lncRNAs analysis pipeline

About the lncRNAs, NONCODE database [59] was used to characterize the annotated lncRNAs in *A. sydowii* H-1 from the assembled transcripts, but none of the transcripts matched the known lncRNAs. To identify novel lncRNAs, we followed the steps below to filter novel lncRNAs from the newly assembled transcripts: Multiple-exon transcripts were considered as being expressed if they had an TPM (Transcripts Per Million) greater than 0.5. For single-exon transcript, more rigorously, TPM should be greater than 2. Those foregone coding-genes or transcripts with size less than 200 nt were filtered out. Then, lncRNAs candidates were identified by CPC2 (Version 0.1) [60], CNCI (Version 3.0)[61], PLEK (Version 1.2) [62] and LGC (Version 1.0) [63]. Candidate transcripts predicted by two or more software as having no coding potential are considered as the lncRNA in *A. sydowii* H-1.

In order to reveal the potential function of lncRNAs, their target genes were predicted in trans and cis-acting. For cis-acting, it refers to lncRNA's action on neighboring target genes. In this study, the coding genes from 100 kb upstream and downstream of the lncRNAs were searched. The trans role refers to the influence of lncRNAs on other genes at expression level. RNAplex [64] and LncTar [65] were used to predict lncRNA target genes in trans-acting. Finally, the Pearson correlation coefficient between lncRNAs and its target genes were calculated by R language. High confidence combination ($|cor| \geq 0.7$ and P-value≤ 0.5) is a certificate to prove the interaction between the lncRNA and the target gene.

Identification of differentially expressed genes (DEGs)

In order to identify DEGs of function genes and lncRNA genes in gene levels, the known gene loci were re-distribution rather than though StringTie software. If the original locus contains newly identified lncRNA, it will be split into two new locus, labeled '-coding' and '-lnc'.The purpose of this is that, first, we focus on the expression at the gene level, and second, Deseq2 software has a better support for the search of differentially expressed genes rather than the expression at the transcriptional level, so the merged gene locus can better identify the differentially expressed genes and reduce the false positive rate. The finally relationship between gene id and transcript id are list at Table S5. The merged gene set were re-quantification using RSEM software [43]. Finally, differential expression analysis between G2 and G8 were performed using the Deseq2 package [66]. P-value ≤ 0.05 and the absolute value of fold change≥ 2 were set as the threshold for significantly differential expressed gene identification.

Phylogenetic Trees of 2-ODD Families

The protein sequences of 2-ODD family, including the candidate transcripts and known anthocyanins-related genes, were aligned using the muscle version 3.8.31 [67] with the default parameters, and the corresponding CDS sequences were back-translated from the corresponding protein sequences. The conserved CDS sequences were extracted by Gblocks method [68], The bootstrap consensus of phylogenetic tree was inferred from 100 replicates. Maximum Likelihood trees were compiled with RAxML version 8.2.7 software [69] and edited with iTOL(https://itol.embl.de).

Real-Time quantitative PCR (RT-qPCR) validation

According to the manufacturer's instructions，total RNA was extracted from 100 mg of fungal mycelia using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed using PrimeScript™RT reagent Kit with gDNA Eraser (TaKaRa). Nine anthocyanins -related genes were selected for RT-qPCR, and the specific RT-qPCR primers were designed by Primer Premier 5 software (Table.S1). Primers for RT-qPCR were synthesized by the Chengdu Qingke Zi Xi Biotechnology Company (Chengdu, China).

By relative quantitative method, each quantitative reaction takes place in a reaction mixture with a total volume of 25 μL, including 12.5 μL 2 × SYBR Premix Ex Taq TM II (TaKaRa), 2 μL diluted cDNA template, 1 μL each primer (10 μM) and 8.5 μL DNA enzyme free water. The amplification was pre-denatured at 95℃ for 30 seconds, then denatured at 95℃ with 40 cycles for 5 seconds, and annealed and extended at 60℃ for 34 seconds. Three technical replicates were tested for each gene, β-tubulin was used as internal reference gene, and 2−ΔΔCT method was used to calculate the relative expression of the gene. All data displays and statistical analyses were performed using GraphPad Prism 5. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, are given in figure legends.

Abbreviations

Pg: pelargonidin

Pn: peonidin

Cy: cyanidin

Mv: malvidin

Pt: petunidin

Dp: delphinidin

PAL: phenylalanine lyase

4CL: 4-coumaroyl CoA ligase

CHI: chalcone isomerase

CHS: chalcone synthase

C4H: cinnamate-4-hydroxylase

F3H: Flavanone -3- hydroxylase

DFR: dihydroflavonol reductase

F3’H: flavonoid 3'- hydroxylase

LDOX: leucoanthocyanidin dioxygenase

UGTs: flavonoid glycosyltransferase

OMT: O-methyltransferase

CPR: cytochrome p450 reductase

G2: The fermentation broth of the second day

G8: The fermentation broth of the 8th day

DNS: Dynitrosalicylic Acid

FTIR: Fourier transform infrared Spectroscopy

tRNAs: transfer RNAs

rRNAs: ribosomal RNAs

NCBI: National Center for Biotechnology Information

NR: Non-redundant protein sequence

KEGG: Pathway Annotation of the Kyoto Encyclopedia of Genes and Genomes

TPM: Transcripts Per Million

DEGs: Differentially expressed genes

Malvidin-3Ga: Malvidin 3-O-galactoside

Peonidin-Mh: Peonidin O-malonylhexoside.

EBGs: early biosynthetic genes

LBGs: late biosynthetic genes

RNAP: RNA polymerase

lncRNA: Long non-coding RNA

2-ODD: 2-oxoglutarate-dependent oxygenases

VIP: Variable Importance in the Projection

FC: Fold Change

Declarations

Acknowledgment

Not Applicable

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Availability of data and materials

Supplementary files are included with this submission and contain all the information needed to reproduce the results of this study. The sequencing data generated in current study are deposited in the NCBI SRA database with the BioProject accession: PRJNA542911.

Authors' contributions

CFB and JZ analyzed the data and drafted the manuscript. QZ performed laboratory experiments. CFB and ZNH participated in the data analysis and revised manuscript. DRQ and HX participated in the sample collection. YC and XYC conceived the study and revised the manuscript. All authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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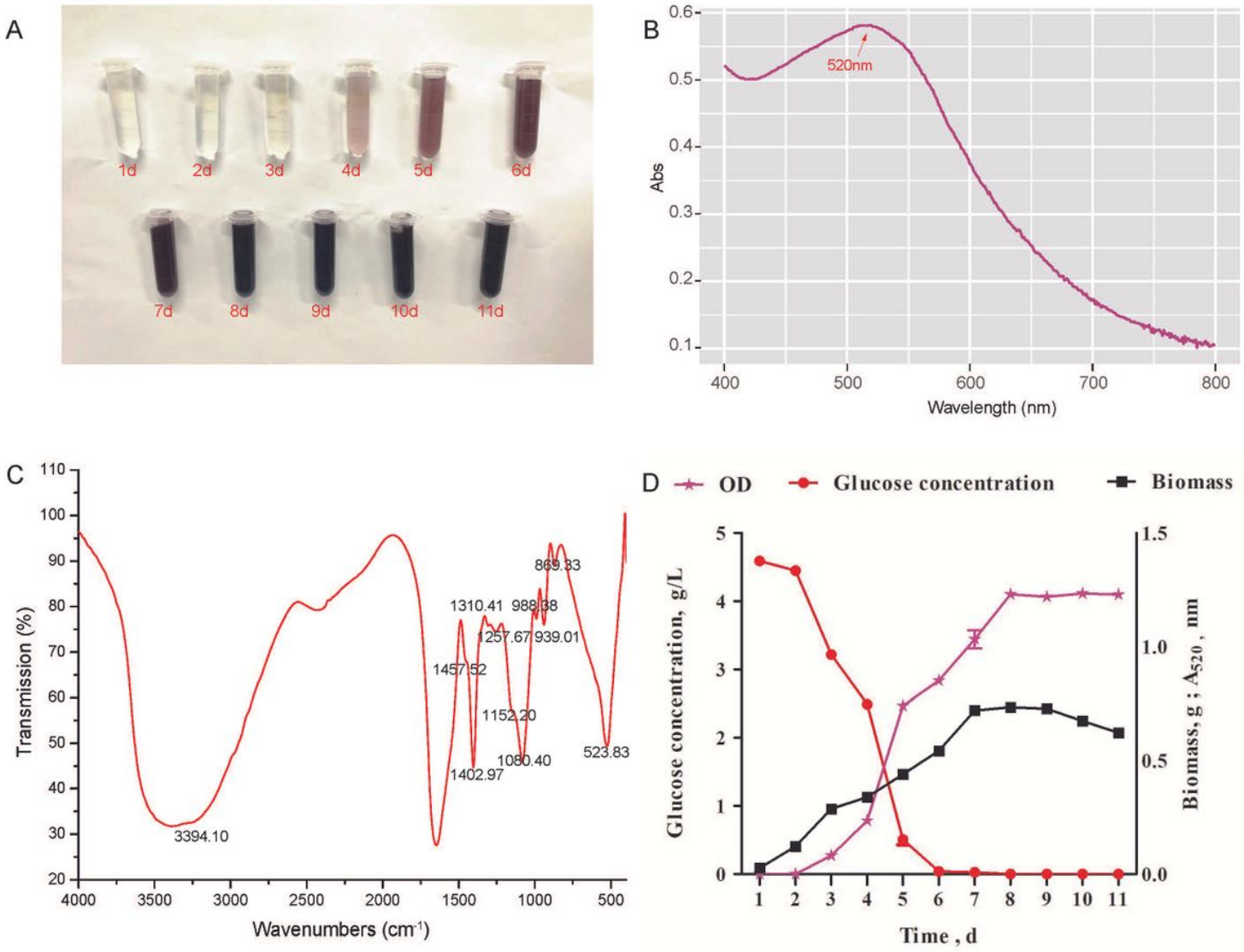
Legends

Figure.1 Relationship between substrate consumption, biomass growth and pigment production. a Color change of A. sydowii H-1 fermentation broth from day 1 to day 11. b Characteristic absorption peak of purple fermentation by spectrophotometer. c Identification of the purple substance by Fourier transform infrared Spectroscopy (FTIR). d Relationship between substrate consumption, biomass growth and pigment production. d: day. Figuer.2 Gene statistics related to anthocyanins metabolism in A. sydowii H-1. a Anthocyanins synthesis pathway. Anthocyanins-related genes can be classified into two major pathways, phenylpropanoid biosynthesis pathway and flavonold biosynthesis pathway, and according to Pelletier et al [44], flavonold biosynthesis pathway can also split into two blocks, anthocyanins early biosynthetic genes (EBGs) and anthocyanins late biosynthetic genes (LBGs). The anthocyanins related compounds identified in A. sydowii H-1 are enclose in purple box. b Heat map of expression of genes

involved in anthocyanins synthesis pathway in A. sydowii H-1. The asterisk indicates a transcript with a significant difference in expression between the two fermentation stages (|fold change| ≥2, P-value≤0.05). Malvidin-3Ga: Malvidin 3-O-galactoside, Peonidin-Mh: Peonidin O-malonylhexoside. Figure.3 Phylogenetic tree of 2-oxoglutarate-dependent oxygenases (2-ODD) gene family. The red dots mark the gene found in A. sydowii H-1. The bamboo green horizontal hexagon and pink octagon represent the relative positions of the common domains of the 2-ODD gene family in this gene, respectively. Figure.4 Genes and lncRNAs related to anthocyanins synthesis pathway. a Venn diagrams illustrating the number of differentially expressed lncRNAs between cis-acting and trans-acting. b Positional relationship between lncRNAs (red dots) and corresponding target genes (purple triangles). The direction of triangles represents the positive and negative of the strand in the genome and the size of the triangle represents the relative size of the locus of the gene. c Bubble plot showing the correlation between anthocyanins compounds and related structural genes or lncRNAs. Only those with a P-value lower than 0.05 are shown. cis: cis-acting, trans: trans-acting, Malvidin-3Ga: Malvidin 3-O-galactoside, Peonidin-Mh: Peonidin O-malonylhexoside. Additional files Additional file 1: Table S1. Primers used in this study. Additional file 2: Table S2. Metabolome data used in this study. Lists of compounds include Ion mode, Rt (min), Molecular Weight (Da), Compounds, Class. MEAN value at G2 and G8, VIP, P-value, log2 fold change. Additional file 3: Table S3. Differentially expressed genes (DEGs) (Pvalue <0.5 and log2FC > 1) in G2 and G8. Lists of DEGs include gene ID, Length, log2FC, P-value, count, annotation. Additional file 4: Table S4. CHS and C4H blast result show that the plant-derived gene may be the reason of the fungus can produce anthocyanins. Lists of blast result include gene symbol, query name, subject name, subject species, identity, alignment length, mismatches length, gap openings, query start, query end, subject start, subject end, e-value and score. Additional file 5: Table S5. The correspondence between gene id and transcript id. Additional file 6: Figure S1. Pearson’s correlation coefficients for each pairwise comparison. Additional file 7: Figure S2. Verification of anthocyanins synthesis related genes by qPCR. The qPCR analyses of eight of function genes and 1 lncRNA gene. Asterisks indicate the significant differences between control and survivor (*: p < 0.05 and **: p < 0.05). Additional file 8: Total transcripts fasta sequences obtained in this study, after assembled by StringTie software and denovo assembled by Trinity software.

Figures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C
Transmission (%)
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1402.97
1310.41
1257.67
1152.20
1080.40
988.38
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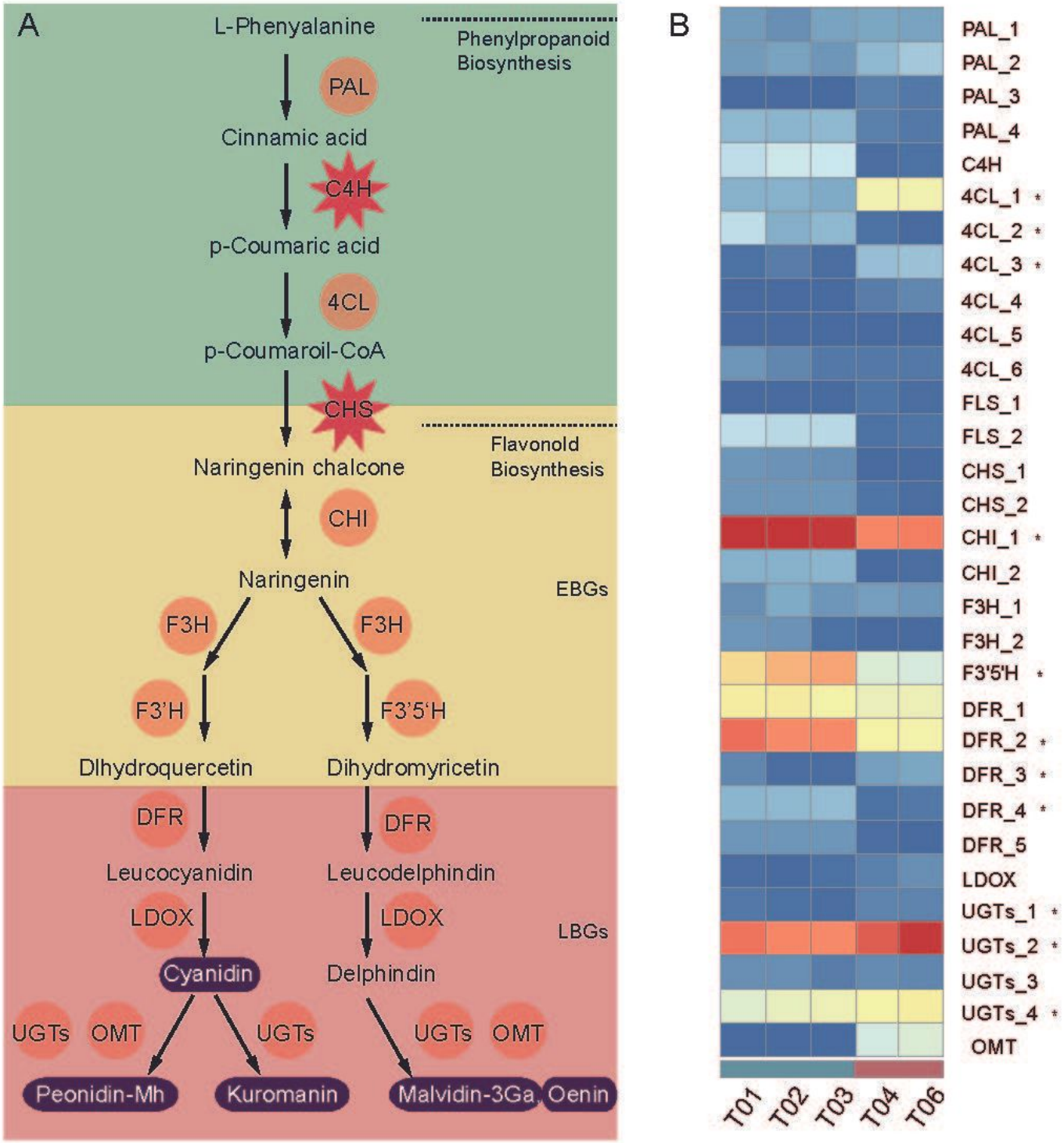
Glucose concentration, g/L
Biomass, g ; A520 , nm
Time , d

Figure 1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Flavonold Biosynthesis
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CHI
Naringenin
EBGs
F3H
F3H
F3'H
F3'5'H
Dlhydroquercetin
Dihydromyricetin
DFR
DFR
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Leucodelphindin
LDOX
LDOX
LBGs
Cyanidin
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T01
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T03
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T06

Figure 2

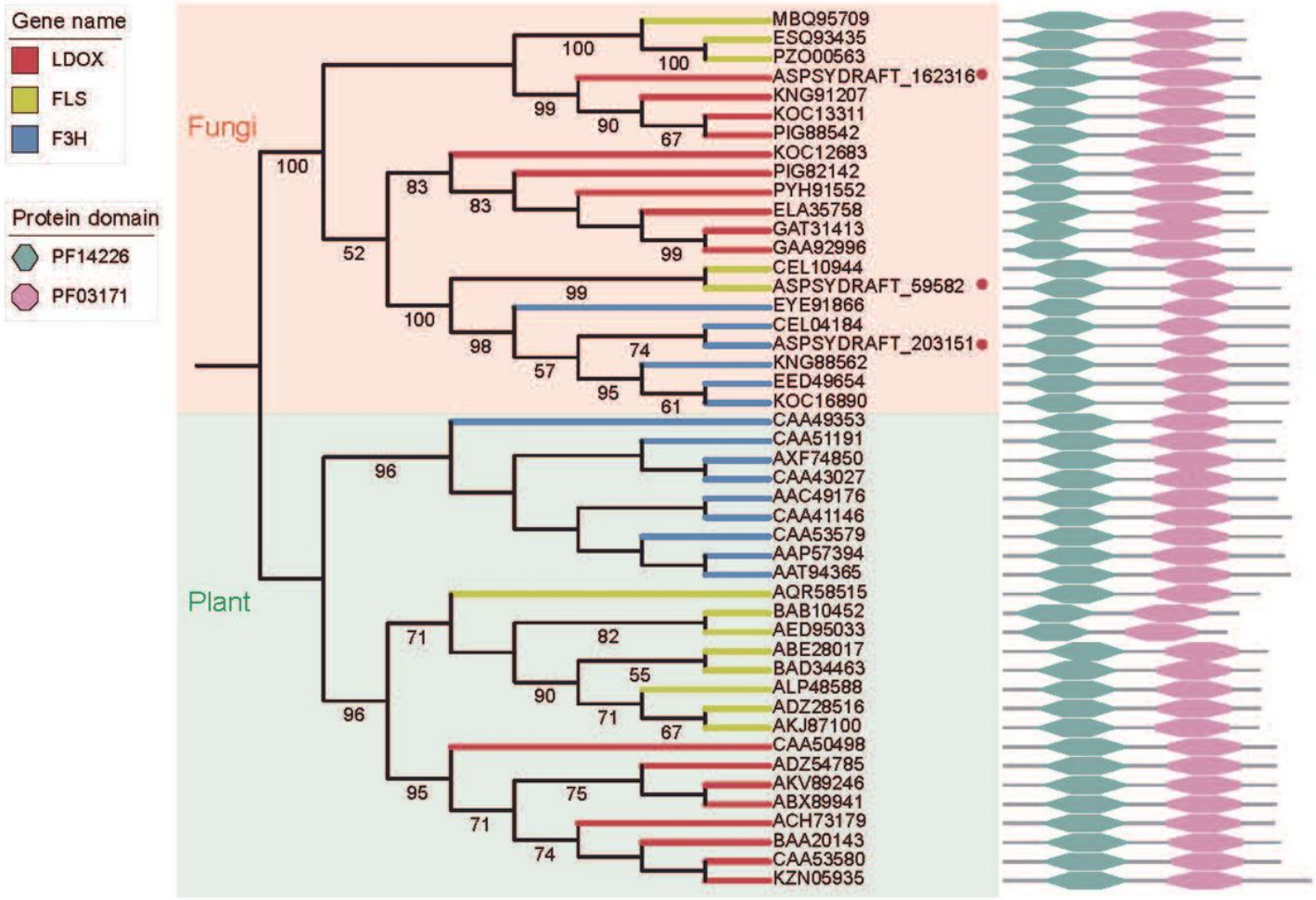
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Figure 3

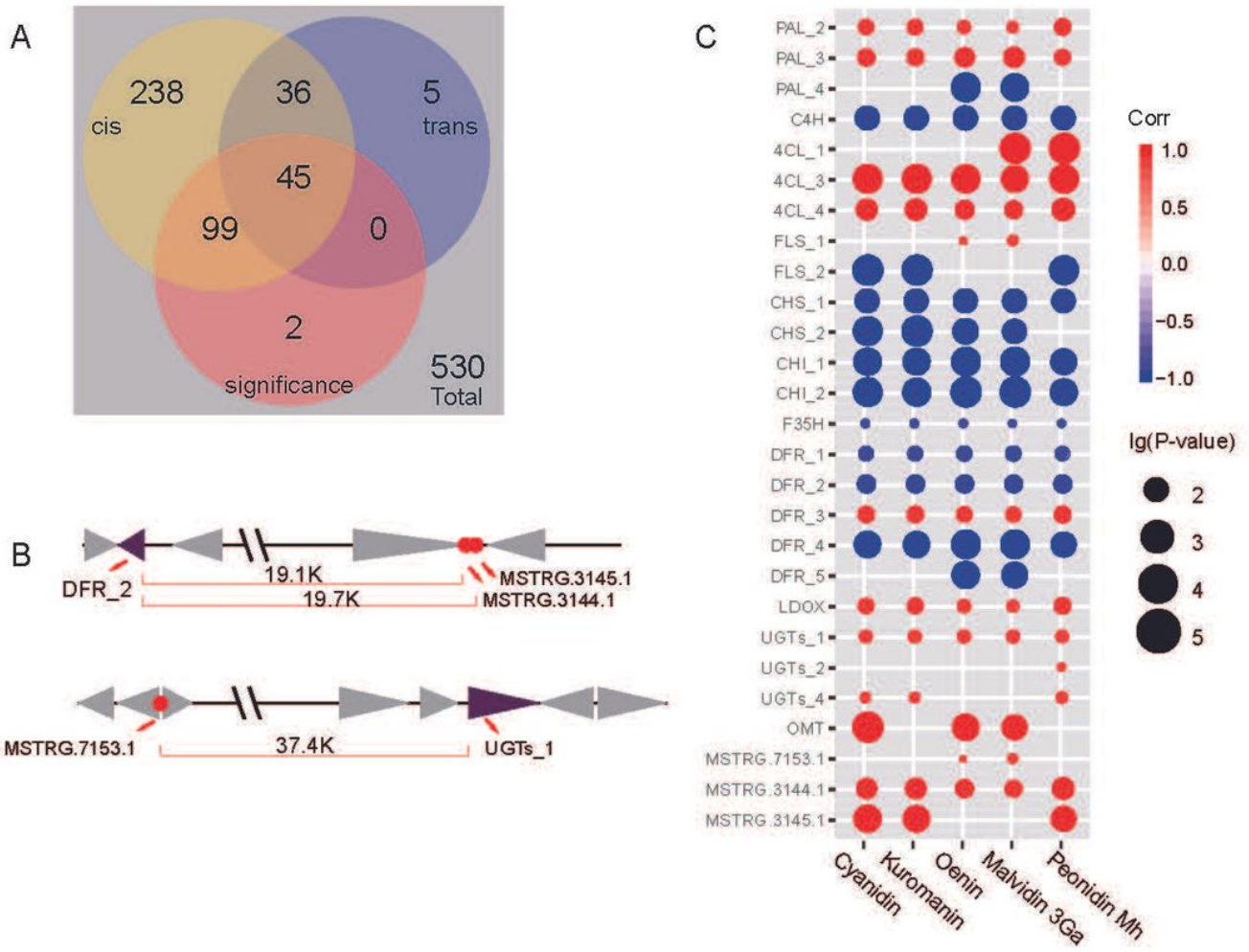

Figure 4

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table.S2metacompounds.xlsx
- table3.lncRNA.xlsx
- Table.S3DEGs.counts.state.xlsx
- Table.S5total.codinglnc.changed.list.xlsx
- Table.S4CHSC4H.blast.result.xlsx
- supplementfile.docx
- table2.anythgenes.xlsx
- table1.assembleState.xlsx
- Table.S1qpcrprimer.xlsx