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Full Length Article

Comparative Transcriptome Analysis of Inbred and Hybrid *Gastrodia elata* (Orchidaceae) to Identify Genes Putatively Involved in Developmental Regulation

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Abstract

Gastrodia elata Bl., the steamed and dried roots known as 'Tianma', is a Chinese traditional medicinal plant belonging to family *Orchidaceae.* Artificial inbred and hybrid Tianma are the main clonal germplasm resource for cultivation, but there are differ in growth rate and the content of active components. Little is known about the molecular mechanisms of growth regulation among inbred and hybrid for this varieties. In this study, RNA sequencing of 12 *G. elata* samples, including inbred Wu, inbred Hong, hybrid Wu-Hong and hybrid Hong-Wu Tianma, was performed using the Illumina HiSeq 2500 platform. The results showed that the *de novo* assembly yielded 383,242 unigenes, a total of 184,706 (48.19%) unigenes was annotated, and 58,065 were assigned to 129 specific metabolic pathways by KEGG. Under the criteria of fold changes ≥2 and q-values < 0.05, approximately 6,122 unigenes were found to be differentially expressed, and three unigenes coding for mannose specific lectin, hexokinase and fructokinase were significantly higher in Hong, Hong-Wu and Hong-Wu Tianma than in Wu Tianma. Consistent with the growth rate, the molecular basis of growth and development regulation in *G. elata* is proposed. The resources generated in this study not only provide new insights into the regulation of growth and development of *G. elata*, but also facilitate future genomic functional studies aiming at producing high-quality *G. elata* germplasm for cultivation. © 2018 Friends Science Publishers

Keywords: *Gastrodia elata*; Transcriptome; Growth and development; Germplasm provenances

Introduction

Gastrodia elata Blume, a Chinese traditional medicine Orchidaceae plant, has been used to treat headaches, vertigo, blackouts, hemiplegia, tetanus and infantile convulsions for thousands of year (Xu and Guo, 2000; Ojemann *et al*., 2006; Jang *et al*., 2015). *G. elata* is widely cultivated and distributed in Sichuan, Yunnan, and Hubei provinces of China, and making this crop an important source of income for farmers. There are four types of *G. elata* based on the colours of the flower and the stem and shape of the corm, which are named *G. elata* B1. *f. elata* (Hong Tianma), *G. elata* B1. *f. Viridis* Makino (Lv Tianma), *G. elata* B1. *f. glauca* S. Chow (Wu Tianma) and *G. elata* B1. *f. flavida* S. Chow (Huang Tianma) (Zhou *et al.*, 1987).

With the exhaustion of wild *G. elata* resources, artificial cultivation technology has gradually been paid attention. In the 1950s, China began to study the domestication of wild *G. elata*, and cultivation reached a peak in the mid-1970s. The cultivation of *G. elata* can be realized by sexual or asexual reproduction. Sexual propagation in *G. elata* is important for promoting its development. Firstly, it addresses the problem of the lack of *G. elata* clonal germplasm resource. Secondly, the regeneration ability, the propagation coefficient, the stress tolerance and the yield will deteriorate during asexual reproduction by *G. elata* tubers. Mima or Baima, the small corms obtained from the germination of the sexual reproduction seed, can significantly improve the propagation coefficient and growth rate and simultaneously maintain vitality for a period of time. Finally, sexual reproduction can produce heterosis through artificial pollination and result in new varieties.

At present, germplasm resources and commercial *G. elata* with high yields and good quality have been obtained by artificial cultivation in some areas. However, the existing studies are limited to the improvement of agricultural characters or the increment of active components, but the basic mechanism of development is not clear, especially the regulation of heterosis in *G. elata*. In our research, a high throughput RNA-Seq transcriptome sequencing technique was used to detect the differences at the transcriptional level

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among two contrasting genotypes of *G. elata* and their hybrids. Functional annotation and enrichment analysis of transcripts was carried out to explore the key regulation genes and metabolite pathways.

Materials and Methods

Plant Materials

The *Gastrodia elata* samples (Mima) analysed in this study were obtained from Sichuan Chi Jian Medicine Technology Co., Ltd. and marked Wu Tianma (Wu ♀ × ♂ Wu, WW), Hong Tianma (Hong ♀ × ♂ Hong, HH), Hong-Wu Tianma (Hong ♀ × ♂ Wu, HW), Wu-Hong Tianma (Wu ♀ × ♂ Hong, WH). For each sample, the transcriptome sequences of three biological replicates were analysed (Fig. 1).

The four kinds of *G. elata* seeds were obtained by self-pollination or hybridization between one Hong Tianma plant and one Wu Tianma plant at sexual reproduction stage, and were cultivated at Guangyuan Tianma cultivation base by members of our research group in May 2016, then collected in January 2017. All the samples were washed with water and 75% ethyl alcohol, and then frozen in liquid nitrogen until RNA isolation.

RNA Isolation and cDNA Library Preparation

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen, Burlington, ON, Canada), and then Oligo (dT) magnetic beads were used to isolate mRNA. By randomly interrupted the mRNA, reverse transcription into cDNA, adaptor ligated, and fragment size selection, a cDNA library was obtained. The library preparations were sequenced on an Illumina Hiseq 2500 platform, and paired-end reads were generated.

Assembly and Annotation

By removing reads containing adapters, reads containing poly-N and low-quality reads from the raw data, clean reads were obtained. The transcriptome assembly was accomplished by Trinity (Grabherr *et al.*, 2011). Unigene function annotation information was obtained by matching against the databases of NR, Pfam, KOG/COG/eggNOG, Swiss-Prot, KEGG, and GO.

Differential Expression Analysis

Gene expression levels were estimated by FPKM for each sample (Trapnell *et al.*, 2010). Differential expression analysis of two groups was performed using the DESeq R package (Anders and Huber, 2010). The resulting P values were adjusted using the Benjamini and Hochberg approach for controlling the false discovery rate (FDR). Differentially expressed unigenes were selected using the criteria that the absolute value of the log2-fold changes (FC) ≥ 1 and FDR < 0.05.

Growth Rate Detection

To evaluate the growth rate of *G. elata*, the fresh and dry weights were determined. From each group, six representative samples were weighed using an analytical balance, and then the average values were calculated. For the determination of dry weight, the samples were dried at 60°C.

Polysaccharide Determination

Approximately 2 g dry *G. elata* powder and 16 mL 80% ethanol were mixed and incubated at 60°C to remove grease and pigment. The residue was then dried, and 80 mL ultrapure water was added. After the mixture had been weighed, it was treated with ultrasound for 34 min at 66°C and then replenished with ultrapure water. The solution was filtered and 0.2 mL filtrate was mixed with 4.8 mL ultrapure water. Then, 1 mL solution was placed in a test tube in an ice bath, 4 mL 0.2% anthrone-sulfuric acid was added, and the mixture was boiled for 10 min. The solution was placed in the dark for 10 min, and the absorbance was measured at 620 nm. The results were calculated using glucose as the standard.

Validation of Gene Expression by QRT-PCR

Quantitative Real-Time PCR (QRT-PCR) was performed to quantify some DEGs based on transcriptome data. The cDNA products derived from mixed samples were used as templates. Their sequences and information were exported from the *G. elata* transcriptome with QRT-PCR primers designed (Table 1).

The quantitative reactions were performed on a Bio-Rad CFX 100 Real-Time PCR System, using SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa). PCR amplifications included the following conditions: one cycle of 95°C for 20 s, 45 cycles of 95°C for 5 s, Tm°C for 20 s and a final melt curve profile of 65–95°C at a rate of 0.5 °C/S. Quantification was determined with the $2^{-\Delta\Delta Ct}$ method. All quantitative PCRs were repeated for three biological and three technical replications.

Results

Detection of Weight and Polysaccharide in *G. elata*

In the same growth environment and time, the weight gain of the sample can represent its growth rate to a certain extent. Hong-Wu Tianma had the maximum weight, both dry and wet, followed by Wu-Hong, Hong, and Wu Tianma (Fig. 2A). The polysaccharide content was highest in Wu-Hong Tianma (16.69 %), next to Wu Tianma (16.25 %) and Hong-Wu Tianma (13.03%), Hong Tianma (11.23%) was the lowest. The results showed that polysaccharide content higher in the *G. elata* obtained by self- or cross-breeding with Wu Tianma as the female parent (Fig. 2B).

Table 1: Primers for QRT-PCR

Unigenes and ID Number	Primers	Sequences (F: 5'→3', R: 5'→3')	Tm (℃)	Length (bp)
mannose specific lectin	F	TAATCGCCTTCGATCTCGCC	60	249
c132247.graph_c0	R	AATGGCCGGTTCTGTTTTGC		
hexokinase	F	TTGAGCTTGCTACCCCCTTC	60	276
c213996.graph_c0	R	AGGGAGGGGATTGATGGGAA		
fructokinase	F	AACACCTTTGACCTGCGGAT	60	113
c218341.graph_c0	R	TGTCGCCATAGCCGTTTCTC		
trehalose 6-phosphate phosphatas	F	GTCGGACAGTCAAAGCCTGA	55	172
c200928.graph_c0	R	CTGCTATGCGCAATGCTGTT		
trehalose 6-phosphate synthase	F	AGTGCACTCTCCTTGTGCTC	55	141
c208237.graph_c1	R	CTGCAGATGGTGTCGGGATT		
actin	F	GGGGACGACCAACAATGCTA	60	153
c193382.graph_c0	R	CCATTCCGCACAGAGTTCCT		

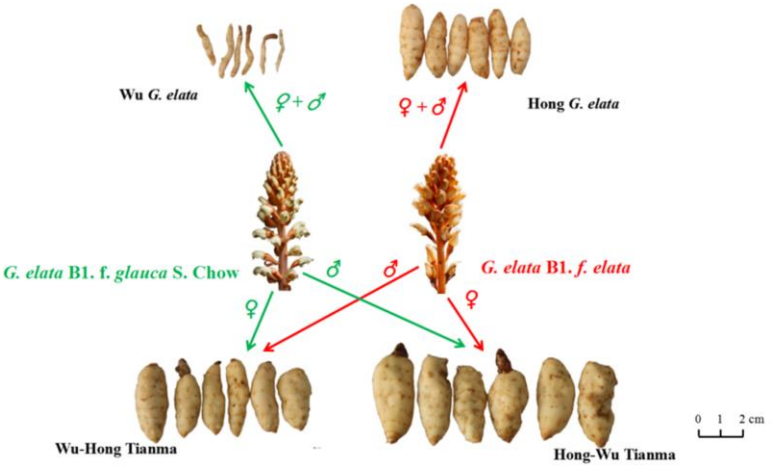

Fig. 1: *Gastrodia elata* samples for the transcriptome sequencing and the principle of each sample parent selection

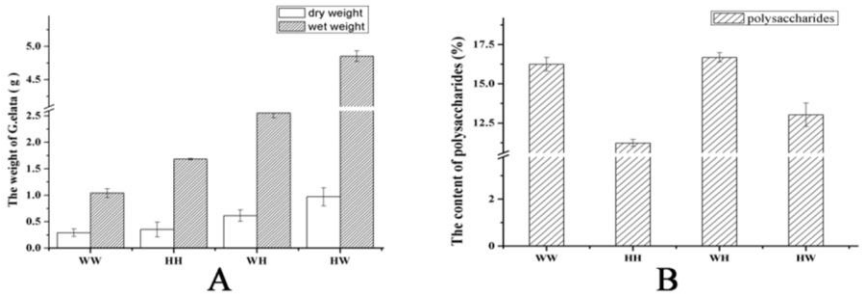

Fig. 2: The content of weight (A) and polysaccharide (B) in four genotypes of *G. elata*

Sequencing and *de novo* Assembly

After sequencing and filtering, total 354,128,614 clean paired-end reads (105,683,557,268 bp) were obtained from the four genotypes of *G. elata* (3 replicates of each sample). A total of 678,678 transcripts and 383,242 unigenes were obtained by *de novo* assembly. As a result, the final N50 lengths of 2,592 and 732 bases, the total lengths of 830,204,890 and 213,939,166 bases were calculated for the transcripts and unigenes, respectively (Fig. 3). All reads were also deposited in the National Center for Biotechnology Information (NCBI) and can be accessed in the Short Read Archive (SRA) under the accession number SRP118053.

Functional Annotation by Sequence Comparison

The alignment and annotation were done according to the sequence similarity search against the public databases, and 184,706 unigenes could be annotated, accounting for 48.19% of the total unigenes (Table 2). The annotation rate for *G. elata*, less than fifty percent, is obviously lower than for other Chinese medicinal herbs. A low annotation rate indicates that the transcriptome of *G. elata* is complex,

Table 2: Summary of the annotations on unigenes of the transcriptomes of *G. elata*

Database	Number of annotated unigenes	Percent of annotated unigenes
COG Annotation	58945	15.38%
GO Annotation	87851	22.92%
KEGG Annotation	58065	15.15%
KOG Annotation	91720	23.93%
Pfam Annotation	98255	25.64%
Swissprot Annotation	71862	18.75%
eggNOG Annotation	157649	41.13%
Nr Annotation	176397	46.03%
All_Annotated	184706	48.19%

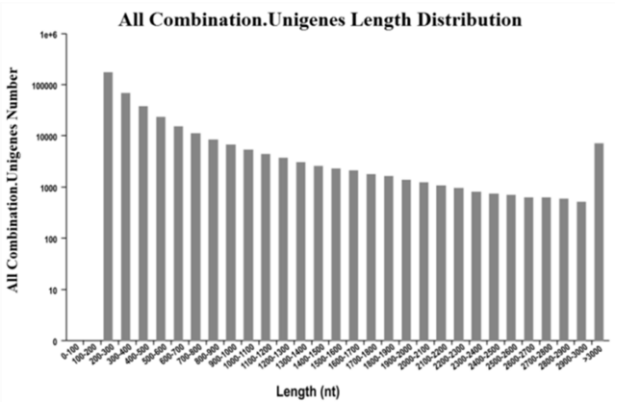

Fig. 3: Length distributions of assembled unigenes

and deep exploration should be given. In the annotation category, the number of KEGG annotations is only 58,065 unigenes (15.15%), showing the lack of information about the secondary metabolite synthesis pathway of *G. elata*, a situation that has greatly limited the progress of research.

Annotation of Differentially Expressed Genes in Four Genotypes of *G. elata*

FPKM analysis based on the total expression quantity showed that a total of 6,122 unigenes were differentially expressed in the four genotypes of *G. elata* (Fig. 4A). Compared with Wu Tianma, the expression of 2486 unigenes increased and 1,668 unigenes decreased in Hong Tianma; 2,636 DEGs were observed in Wu-Hong Tianma, with 926 unigenes up-regulated and 1,710 unigenes down-regulated; Hong-Wu Tianma contained 705 DEGs, 178 up-regulated unigenes and 527 down-regulated unigenes. Compared with Hong Tianma, 1564, 1816 DEGs were identified in Hong-Wu and Wu-Hong Tianma, respectively. And compared with Hong-Wu Tianma, 810 DEGs existed in Wu-Hong Tianma with 598 unigenes up-regulated, 212 unigenes down-regulated (Fig. 4B).

Identification of Development-relevant Regulatory Signals in *G. elata*

Normal swelling of the root tuber of *G. elata* directly determines its quality and yield. The results of our study

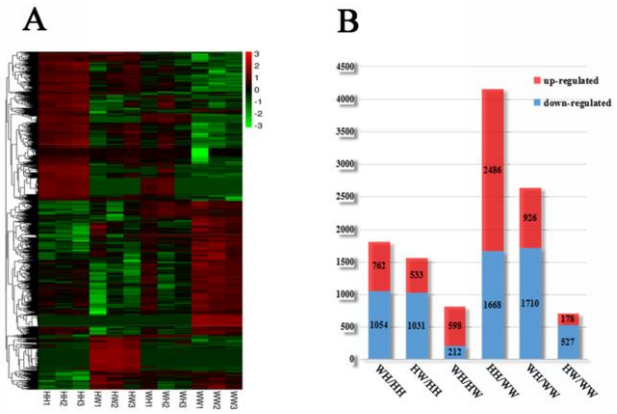

Fig. 4: The expression of all differentially expressed genes (DEGs) among four genotypes of *G. elata*

Note: (A) The heat-map of the total DEGs among four kinds of *G. elata*. Samples are displayed below the heat maps. Color scale indicates the expression levels from low (green) to high (red). (B) The numbers of up- and down-regulated genes in all 6 comparisons among four genotypes of *G. elata*

suggest that the growth rates of hybrid *G. elata* were faster than those of the inbred plants and that the hybrids had a certain degree of heterosis.

The growth rate of Hong-Wu Tianma was the fastest and the Wu Tianma was the slowest from the root volume (or weight). And totally 178 unigenes up-regulated in Hong-Wu Tianma, were grouped in "replication, recombination, repair" and other biological processes related to development. "Starch and sucrose metabolism" was the most abundant pathway in KEGG enrichment, including 4 related signalling factors, c200928.graph_c0 (trehalose 6-phosphate phosphatase, TPP), c208237.graph_c1 (trehalose 6-phosphate synthase, TPS), c213996.graph_c0 (hexokinase, HXK), and c218341.graph_c0 (fructokinase, FRK). The most significant unigene among the 487 DEGs expressed both in Hong-Wu and Wu Tianma is c132247.graph_c0 (log2FC=13.66). The FPKM values are 10093.4176 and 0.8039 in Hong-Wu and Wu Tianma, respectively. The protein is a mannose specific lectin and can bind specifically to alpha-D-mannose with 1 ~ 3 active binding sites in each subunit, which is may play an important role in plant resistance to pathogenic microorganisms, pests and phytophagous animal.

Interestingly, KEGG enrichment analysis between Hong-Wu and Wu Tianma showed that the differentially expressed genes were mainly concentrated in "Photosynthesis", "Photosynthesis antenna proteins", "Carbon fixation in photosynthetic organisms", "Carbon metabolism", and most of them were highly expressed in Wu Tianma (Fig. 5). Notably, among the 436 common DEGs of Wu Tianma compared with other three, 18, 17, 10 and 3 unigenes were annotated to "photosynthesis", "carbon fixation in photosynthesis organisms", "photosynthesis-antenna proteins" and "porphyrin and chlorophyll metabolism", respectively (Table 3).

Table 3: photosynthesis related unigenes and its expression in Wu Tianma

#ID	FPKM	Definition
c121122.graph_c0	4.013069991	photosystem I subunit X
c121299.graph_c0	3.884865499	photosystem II 22kDa protein
c121327.graph_c0	2.977291926	photosystem I subunit PsaN
c125426.graph_c0	1.609627686	photosystem II PsbY protein
c137997.graph_c0	3.894968152	photosystem I subunit II
c139906.graph_c0	3.038894677	photosystem I subunit V
c141193.graph_c0	6.217172359	photosystem II oxygen-evolving enhancer protein 2
c151442.graph_c1	3.599943802	cytochrome b6-f complex iron-sulfur subunit
c173421.graph_c1	10.76310055	photosystem II 10kDa protein
c209464.graph_c1	6.071619427	photosystem II oxygen-evolving enhancer protein 1
c218961.graph_c0	3.324763017	photosystem II PsbW protein
c218998.graph_c0	3.264116349	photosystem I subunit PsaO
c219028.graph_c0	3.366784639	photosystem II oxygen-evolving enhancer protein 3
c219058.graph_c0	4.293038248	plastocyanin
c219068.graph_c0	5.567202684	photosystem I subunit XI
c219092.graph_c0	4.129738576	photosystem I subunit III
c117610.graph_c0	3.518348191	light-harvesting complex I chlorophyll a/b binding protein 4
c138761.graph_c0	3.576961103	light-harvesting complex I chlorophyll a/b binding protein 3
c166975.graph_c0	1.151367199	light-harvesting complex II chlorophyll a/b binding protein 5
c187974.graph_c1	2.525363231	light-harvesting complex II chlorophyll a/b binding protein 6
c195868.graph_c0	5.952494803	light-harvesting complex I chlorophyll a/b binding protein 2
c207058.graph_c1	32.12405986	light-harvesting complex II chlorophyll a/b binding protein 2
c218985.graph_c0	4.357810931	light-harvesting complex I chlorophyll a/b binding protein 1
c219065.graph_c0	4.188895034	light-harvesting complex II chlorophyll a/b binding protein 5
c219097.graph_c0	9.435204023	light-harvesting complex II chlorophyll a/b binding protein 4
c219295.graph_c0	1.701108924	light-harvesting complex II chlorophyll a/b binding protein 4

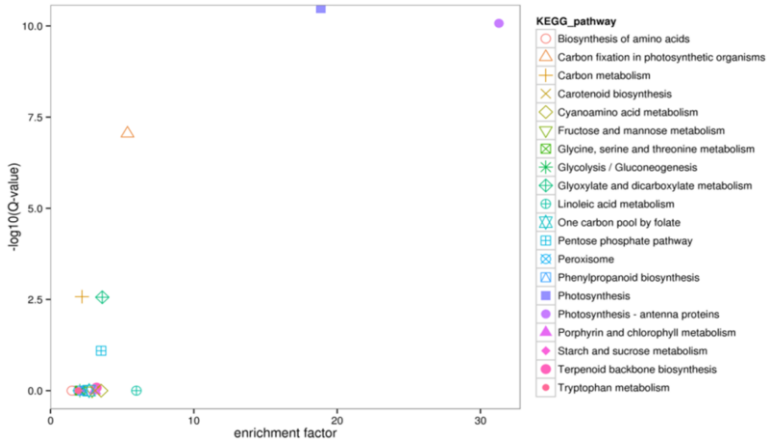

Fig. 5: KEGG Enrichment analysis between Hong-Wu and Wu Tianma

Note: each graphical in the figure representation of a KEGG channel, and the channel name showed in the right side. The abscissa is the enrichment factor, and the greater the enrichment factor, the more significant the enrichment level of differentially expressed genes in the pathway. The ordinate is log10 (Q value), which Q value for multiple hypothesis testing corrected P value, and the greater the ordinate, the more reliable the significance of the differentially expressed genes in the pathway is

The range of photosynthesis related proteins included light-harvesting complex I, chlorophyll a/b binding protein, photosystem subunit, photosystem II oxygen-evolving enhancer protein 2, and plastocyanin.

Validation of Some Important Genes Participating in the Regulatory Signals

To validate the differential expression of growth-related unigenes, we analysed the expression patterns of 5 important gene sequences in the *G. elata* transcriptome by using real-time PCR (QRT-PCR). The results showed that all the tested genes were transcribed in the 4 kinds of *G. elata* samples analysed (Fig. 6). The expression trends of the QRT-PCR analysis were consistent with the RNA-Seq data, indicating that the transcriptome data obtained in this study had good accuracy and reference value.

Discussion

Differential analysis of transcriptome data suggest that the growth and development of *G. elata* may be related to fungal interaction, energy metabolism and environmental

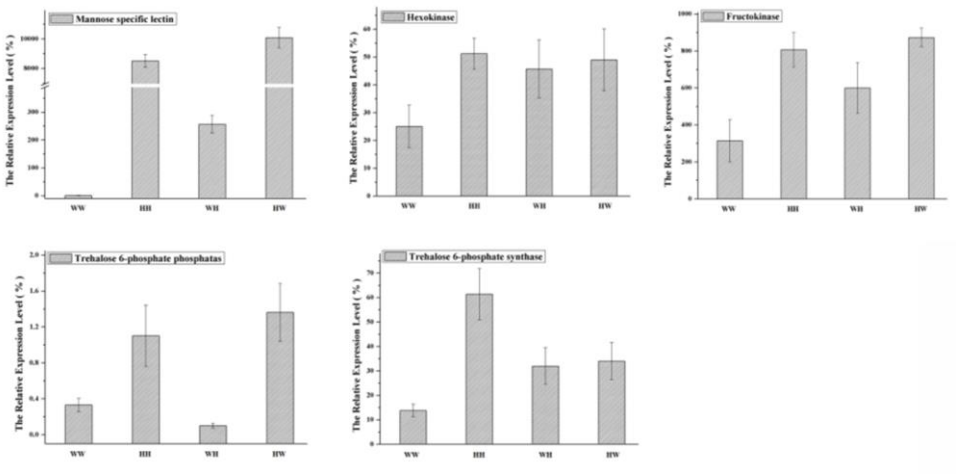

Fig. 6: QRT-PCR confirmation of selected genes

stress. The expression or accumulation of antifungal genes and proteins directly affects the activity both *G. elata* and the *Armillaria* fungus. Only if *G. elata* can control the *Armillaria* invasion or not, then can ensure its development by absorbing and decomposing the fungal rhizomorph as a source of nutrition. In fact, *G. elata* grows in a symbiotic relationship with two compatible mycorrhizal fungi, *Mycena* spp. and *Armillaria mellea*, during seed germination and vegetative growth, respectively (Tsai *et al.*, 2016; Zeng *et al.*, 2017). From seed germination to tuber maturation, *G. elata* relies on the invasive rhizomorph of *A. mellea* to provide nutrition. Different developmental stages have different reactions to *A. mellea*, including rejection, control and opening, determined by the increase and decrease of lysozyme (Zhou *et al*., 1987). In 1988, Kunming plant researchers isolated a protein named gastrodia antifungal protein (GAFP, later renamed gastrodianin) from Hong Tianma (Hu *et al.*, 1988). This protein can inhibit *A. mellea* and *Trichoderma viride* in vitro and reduce *A. mellea* dissemination into *G. elata* corms. This result indicated that the accumulation of gastrodinin plays an important role in resistance to *A. mellea*. Later, gastrodianin gene has been cloned from Hong Tianma, Huang Tianma and Wu Tianma, and gastrodianin is considered belong to the mannose binding protein (MBP) superfamily according to the protein similarity comparison (Xu *et al.*, 1998; Wang *et al.*, 2001, 2007). The GAFP-1 isolated from *G. elata* was transferred into tobacco, and the transgenic tobacco showed resistance to fungi (Cox *et al.*, 2006). The c132247.graph_c0 gene coded for mannose specific lectin is therefore considered to play a defensive role against the *Armillaria* fungus in Hong-Wu Tianma, and it is conducive to the growth and development of *G. elata*, which acquires its nutrition primarily from *A. mellea*.

Sufficient energy is another factor in plant development. HXK and FRK belong to the generalized hexokinases, which were significantly higher in Hong-Wu Tianma. Hexokinases are the key enzymes of respiratory metabolism in plants and can phosphorylate hexoses. In recent years, studies of the molecular mechanisms of plant sugar signalling showed that as an intracellular sugar sensor, HXK plays an important role in glucose signal transduction, and this signal transduction pathway regulates plant growth and gene expression (Smeekens, 2000). Whether growth is promoted or inhibited depends on the intrinsic glucose levels and glucose sensitivity of the plants (Ramon *et al.*, 2008). In wild-type plants, high light conditions (200–300 μE) can boost photosynthesis and sugar production, leading to accelerated growth and early leaf senescence. However, gin2 (HXK1 mutants) plants remain small and dark green and show little cell expansion, and the distance between trichomes is short (Moore *et al.*, 2003). *G. elata* has a close relationship with symbiotic *Armillaria* and cannot carry out photosynthesis in the underground. Therefore, the decreased photosynthetic rate caused by a high expression level of HXP in *G. elata* cannot affect its development. The expression level of HXP and FRK was higher in Hong-Wu Tianma than in Wu Tianma, which may be associated with the physiological activities of the enzymes. With the rapid growth and strong respiration of Hong-Wu Tianma, the high level of expression of hexokinase can regulate glycolysis and provide energy for its development.

The ability to adapt to the environment reflects the evolutionary trend of the survival of the fittest. TPS and TPP, and even the mannose specific lectin in Hong-Wu Tianma are coded by multiple stress responsive genes and reflect the adaptability of *G. elata* to the environment. TPS and TPP are the key enzymes in the biosynthesis of trehalose, and trehalose can be stored and transported to protect organisms from environmental stress. Jang transferred a gene encoding a bifunctional fusion (TPSP) of the TPS and TPP of *Escherichia coli* into rice, and the accumulation of trehalose in the transgenic rice was significantly higher than in the original plants, which resulted in increased tolerance to drought, salt, and cold (Jang *et al.*, 2003).

Interestingly, previous study showed that *G. elata*, which is an achlorophyllous orchid plant, was completely dependent on its fungal partners throughout its lifetime (Park *et al.*, 2012). The reason why chlorophyll related genes were detected in samples of Wu Tianma is not clear, but some scholars speculate that *G. elata* may have originated from the relatively primitive green orchids in ancient times and were an autotrophic plant originally. Some individuals may have had an advantage in the accumulation of antifungal substances in the long-term struggle with *Armillaria*, and thus, *G. elata* may have gradually changed its autotrophic style and formed a special relationship with *Armillaria*. The chlorophyll related signal detected in this study is the inherent property of Wu Tianma, or due to individual differences in sampling or to the experimental treatment, which needs further validation. In the dark, the thylakoid membranes of the chloroplast rupture and fuse with each other to form a typical lamellar structure, named the leucoplast. The leucoplast has the function of storing starch granules, and may be related to the high content of polysaccharides in Wu Tianma.

Conclusion

There existed certain hybridization advantage for this species, and the transcriptional level of the four genotypes of *G. elata* was different. Three key genes may associated with growth regulation, putatively encoding mannose specific lectin and hexokinase, have been identified, which may help to elucidate the physiological functions of heterotrophic plants. In addition, the transcriptome data provided the foundation for future studies of gene expression, functional annotation and metabolic mechanisms in *G. elata*.

Acknowledgements

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