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The Clone and Analysis of the Gene of 14-3-3 Protein, A Regulating Protein from *Ganoderma lucidum*

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Abstract: It was found in our previous study that the expression of *Ganoderma lucidum* 14-3-3 protein gene (Gl14-3-3) was significantly changed during the growth of *G. lucidum* by different light quality. In eukaryotes, 14-3-3 protein is a highly conserved regulatory protein encoded by the gene family which plays a major role in protein-protein interaction and participates in apoptosis and division, signal transduction. As an important regulatory protein dispersed in the organism, the regulation of Gl14-3-3 on the growth and development of *G. lucidum* is of great significance for the optimization of environmental conditions in cultivation. In this study, the cDNA sequence of Gl14-3-3 was cloned and its full length was 1056 bp, including a complete open reading frame of 774 bp. The protein encoded by this gene is predicted to consist of 257 amino acids with a relative molecular mass of 29030.76 Da. The protein contains two major functional domains, Src homeodomain 2 (SH2) and cAMP-dependent protein kinase domain (protein kinase A, PKA). Homologous alignment analysis revealed that it had high homology with the 14-3-3 protein of the basidiomycete fungus. Real-time quantitative PCR analysis was used to discover the expression of Gl14-3-3 of

mycelium between the blue light and dark, different temperature conditions, respectively. The results showed that the relative expression of Gl14-3-3 by the blue light was higher than that with dark. The tendency of the expression of Gl14-3-3 by the blue light was not the same as that with dark. As to the different temperature treatments, the relative expression of Gl14-3-3 was lower at low temperature. These results suggested that Gl14-3-3 might regulate the growth and development by responding to different environmental signals discriminatively. With a deeper understanding in the mechanism of Gl14-3-3, the environmental condition could be improved and designed accurately for better quality and yield in the facility cultivation of *G. lucidum*. The results of the study could also be used for reference on other edible fungi.

Introduction

G. lucidum is the famous Chinese traditional medicinal fungus with many chemical constituents, such as polysaccharides[1], triterpenoids[2], nucleosides, alkaloids and so on. *G. lucidum* has remarkable effects in enhancing human immunity, regulating blood sugar, controlling blood pressure and assisting tumor radiotherapy and chemotherapy[3]. The source of *G. lucidum* in China mainly depends on facility cultivation. *G. lucidum* is affected by environmental factors such as water, light, and temperature during growth and development. Environmental factors often become stressors that exacerbate growth and thus change quality and yield. It is of great significance to study the resistance and good agronomic traits of *G. lucidum* that regulate the growth and differentiation.

14-3-3 protein was first isolated from the mammalian brain[5]. In eukaryotes, 14-3-3 protein is a highly conserved regulatory protein encoded by the gene family which plays a major role in protein-protein interaction and participates in apoptosis and division, signal transduction. They participate in almost all biological activities[6,7,8]; They are involved in cell signalling, regulation of cell cycle progression, intracellular trafficking/targeting, cytoskeletal structure and transcription, protein transmembrane transport, gene transcription, polysaccharide synthesis, and so on. In Saccharomyces cerevisiae, 271 proteins may interact with 14-3-3 proteins[9]. As to edible fungus, it was found that 14-3-3 protein could interact with Adenylyl Cyclaseassociated Protein (cap) in *Lentinus edodes*[10], with protein containing WD domain in *Phanerochaete chrysosporium*[11]. In *Hypsizigus marmoreus*, it was found that the expression of 14-3-3 protein increased during hypothermia treatment[12].

The existence of 14-3-3 protein and its significantly different expression in*G. lucidum* induced by different light quality were first reported by our lab[13]. On this basis, the 14-3-3 protein gene of *G. lucidum* was cloned utilized by the known *G. lucidum* genome. Furtherly the regulation of 14-3-3 protein was studied by analyzing the expression level of 14-3-3 protein gene of *G. lucidum* with blue light and different temperature. It was expected that the study on the regulation of 14-3-3 protein during the growth and development could reveal the resistance mechanism of *G. lucidum* and help to improve the condition of cultivation.

1 Materials and Methods

1.1 Strains, culture conditions and genetic techniques

The strain*G. lucidum* S3 was from biological fermentation laboratory at Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical Collage as described previously[14]. LED as light source, the range of blue light wavelength was 430～480 nm. The mycelium of *G. lucidum* cultured in the incubator at 15℃, 20℃, 25℃ and 30℃, respectively.

From the 6th day, the sample was taken every 2 days until the 10th day in triplicate.

1.2 Gl14-3-3 gene cloning and sequencing

The nucleotide sequences and amino acids of Gl14-3-3 gene were obtained from the *G. lucidum* Information Resource (http://www.herbalgenomics.org/galu). Genomic DNA was prepared using the Plant Genomic DNA Kit (TIANGEN, China). PCR amplification was performed in a MyGeneTM Series Peltier Thermal Cycler (Long Gene, China), overall length primers Gl14-3-3-F/R (Table 1) were designed with Primer Premier 5.0. PCR products were gel-purified, cloned, and then sequenced by the Sangon Biotech (Beijing, China).

1.3 Sequence annotation, phylogenetic analysis, and motif detection

The retrieved sequences were used for gene model prediction on the GENSCAN web server (http://genes.mit.edu/ GENSCAN.html). The theoretical isoelectric point (pI) and molecular weight (Mw) were predicted using the Compute pI/Mw tool on the Protparam (http://web.expasy.org/protparam/). Motif scan analysis was with the program Simple Modular Architecture Research Tool (SMART) (http://smart.emblheidelberg.de/). Phylogenetic trees were constructed using MEGA 5.0 with the neighbor-joining method. Bootstrap test was replicated 1000 times.

1.4 RNA extraction and quantitative realtime reverse transcription-PCR (qRT-PCR)

Total RNA was extracted using the Quick RNA Isolation Kit (Huayueyang, China). RNA integrity was analyzed on a 1.0% agarose gel. RNA quantity was determined using a NanoDrop2000C Spectrophotometer (ThermoScientific, USA). cDNA synthesis was carried out using Fast Quant RTKit (TIANGEN, China). Quantitative real-time PCR were performed using the Super Real PreMixPlus(SYBRGreen) kit(TIANGEN, China) and carried out in triplicate for each sample. Gene-specific primers Gl14-3-3-F and Gl14-3-3-R (Table 1) were designed using Primer Premier 5.0. The expression of Gpd (glyceraldehyde-3-phosphate dehydrogenase, GenBank accession number: DQ404343.1) was stable under the experimental conditions and was selected as a reference gene as described previously[15]. SYBR Green RTPCR reactions of samples were performed using 2 × SuperReal PreMix (TIANGEN, China) in 10 μl volumes containing 1 μl of cDNA template and 10 μM forward and reverse primers. The program used was: 15min at 95 ℃; 40 cycles of 98 ℃ (15s), 60 ℃ (30s); with a melting curve from 60 ℃ to 95 ℃ (increment 5 ℃/s). Reactions were run in BioRadCFX96(BioRad, USA). Three independent biological replicates were performed. Statistical analysis was carried out as described. $2^{-\Delta\Delta Cq}$ was used to achieve results for relative quantification. For statistical analysis, ANOVA (analysis of variance) was calculated by using SPSS (Version 17.0, IBM, USA).

Table 1 PCR primer used in this study

Primer name	Sequence
Gl14-3-3-F	TCTCCCCTCGTCCTCGTGCTCG
Gl14-3-3-R	TACACCGAGACGGGAAAACTGC
14-3-3-F	CATCATGCAACTCCTCCGTG
14-3-3-R	GATGCGGATGATGGATGAGC
gpd-F	GATGAAGGACTGGCGTGGT
gpd-R	CCGTTGAGGCTGGGAATGAC

2 Results

2.1 Cloning and nucleotide sequence analysis

The full length of the Gl14-3-3 cDNA sequence of *G. lucidum* was 1056 BP (Fig. 4.2). ORF Finder analysis showed that the sequence contained a complete open reading frame 774 BP (70-843 bp), containing the starting codon ATG and the terminating codon TAA, encoding 257 amino acids.

The relative molecular weight, theoretical isoelectric point, hydrophobicity, instability coefficient and aliphatic amino acid index of GL14-3-3 were predicted by ProtParam program in ExPASy . The deduced relative molecular weight of Gl14-3-3 protein is 29030.76 Da, pI 4.79. The positive and negative charge residues were 32 and 50, respectively, belonging to acidic proteins. The predicted instability coefficient is 49.46 which indicates that the protein is an unstable protein. The aliphatic amino acid index was 82.49. The hydrophilicity of *G. lucidum* was -0.539, indicating that the predicted *G. lucidum* GL14-3-3 protein was hydrophilic.

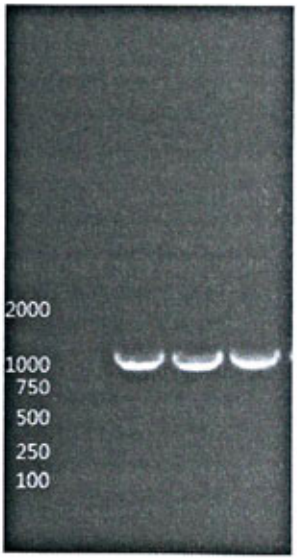

Fig. 2 Full-length PCR bands of Gl14-3-3

The results of the SMART analysis showed that the amino acid sequence encodes a 14-3-3 conserved domain, which was a serine / threonine binding protein commonly found in yeast, protozoa and mammals. It contained 14-3-3 protein superfamily domain and a specific site of fungal 14-3-3 protein. The amino acid sequences which were easy to be phosphorylated by specific protease was predicted online by Scansite3. The predicted results indicate that the amino acid sequence of this protein was the typical domain of 14-3-3 protein. It contained two major functional domains (Fig. 4), one of which is Src homologous domain 2 (SH2). SH2 was a highly conserved and non-catalytic domain of intracellular signal transduction molecules, which can bind to phosphorylated tyrosine and mainly mediates the interaction between proteins, participates in the formation of signal complexes and constitutes signal transduction chains[16]. The other was the cAMP dependent protein kinase domain (Protein kinaseA, PKA), whose main function was to transfer phosphate groups from ATP to the serine or threonine residues of specific proteins for phosphorylation. Proteins phosphorylated by protein kinases regulate the activity of target proteins[17]. These two domains play an important role in the interaction of 14-3-3 proteins with other phosphorylated proteins.

2.2 Phylogenetic analysis of Gl14-3-3

BLAST database analysis showed that the predicted Gl14-3-3 protein had high homology with other fungal 14-3-3 proteins. Phylogenetic analysis of 14-3-3 amino acid sequences of 18 fungi by MEGA6.0 software Neighbor-Joining (Fig. 5) showed that the Gl14-3-3 was closely related to *Dichomitus squalens*, which belongs to the basidiomycetes. The sequence of 14-3-3 is divided into two categories: basidiomycetes and ascomycetes, including Gl14-3-3. The others belong to few orders of *Deuteromycotina*.

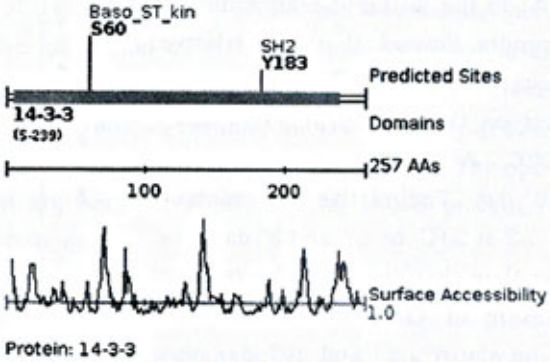

Fig. 4 Functional structure domain prediction of Gl14-3-3

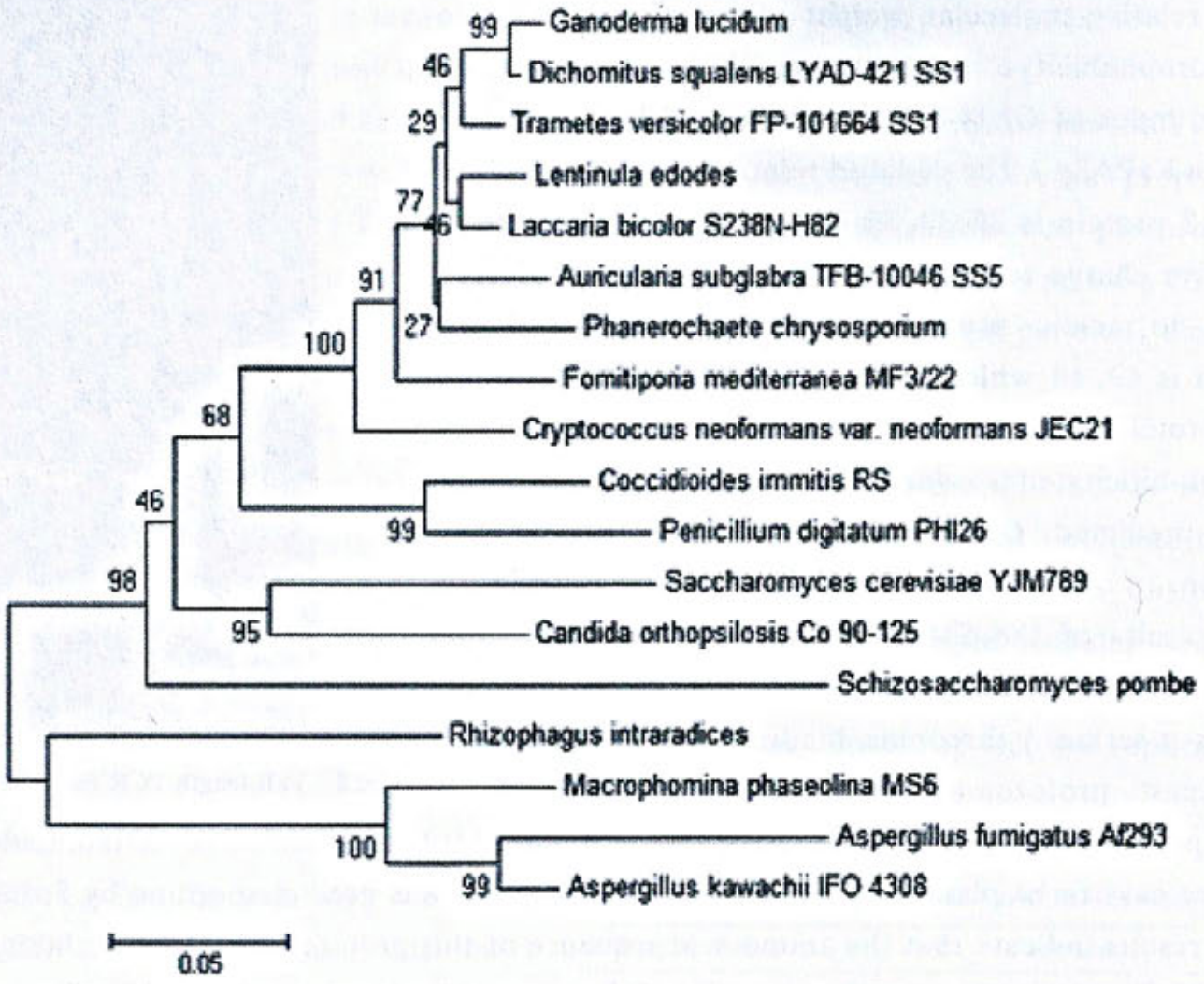

Fig. 5　The phylogenetic tree of Gl14-3-3 from G. lucidum with other fungi

2.3 Relative expression of Gl14-3-3

As to the influence of light, the relative expression of Gl14-3-3 in the mycelium with bluelight increased during 10 days, and reached the highest level in the 10th day. The relative expression of the control(without light, i.e. dark) increased first and then decreased, reached the highest level on the 8th day. The relative expression of Gl14-3-3 with blue light was higher in 6 days, 8 days and 10 days than that of control. To the 8th and 10th day, the relative expression of Gl14-3-3 with blue light was 2.1 and 4.8 times of that of the control(Figure 6).

As to the different temperatures, the results showed that the relative expression of Gl14-3-3 were the highest on the 8th day at 30℃, 25℃ and 20℃. At 15℃, the highest was on the 10th day. The relative expression of Gl14-3-3 at 30℃ on 6th and 8th day was higher than that at 25℃. The relative expression of Gl14-3-3 at 20℃ and 15℃ on the 6th, 8th and 10th day was lower than that at 25℃. On the 8th day, the relative expression of Gl14-3-3 at 20℃, at 15℃ was two fifth, one fifth of that at 25℃, respectively (Fig.7).

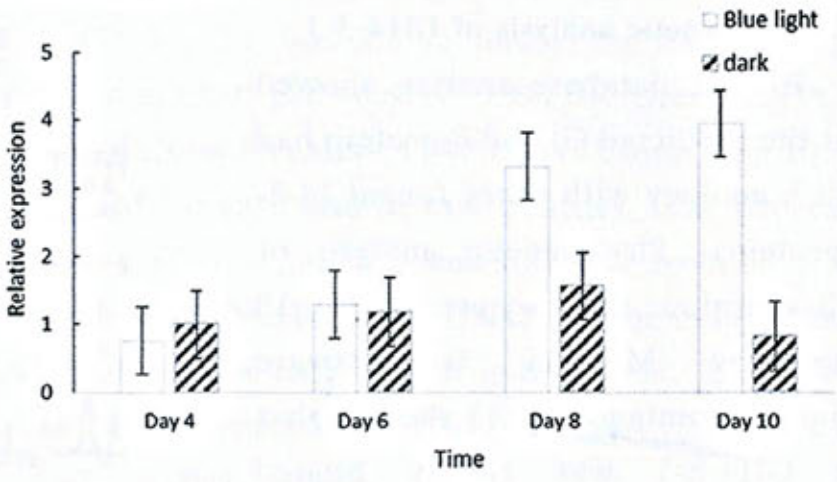

Fig. 6　The relative expression of Gl14-3-3 gene with blue light and dark

3　Discussion

14-3-3 protein is a signal regulator widely present in eukaryotes. This sequence is highly

conserved and is encoded by a gene family in most species[18]. In this study, 1026 BP was used to clone the putative 14-3-3 protein gene Gl14-3-3 of *G. lucidum* S3, which contains a complete open reading frame of 774 bp encoding 257 amino acids.

The Gl14-3-3 protein was found to contain 14-3-3 protein conserved domain containing a 14-3-3 protein superfamily domain and a specific site for the fungal 14-3-3 protein. NCBI sequence alignment and domain analysis showed that Gl14-3-3 had high homology with 14-3-3 protein sequence of basidiomycetes. Two major functional domains SH2 and PKA, were found to play an important role in the interaction between 14-3-3 proteins and other phosphorylated proteins. 14-3-3 proteins interact with phosphorylated proteins. And it plays a regulatory role in various cellular functions and physiological processes[19].

In eukaryotes, 14-3-3 protein is involved in signal transduction, induced by hypothermia, hypoxia and high salt,, regulating growth and development, signal transduction and so on. It has been shown that the 14-3-3 protein of the fungi *Ustilago maydis* was involved in the control of cell polarity and regulation of cell cycle [20]. The analysis of the expression of Gl14-3-3 gene of *G. lucidum* showed that the highest value of Gl14-3-3 gene expression with blue light was not the same as that with dark, which indicated that the trend of Gl14-3-3 gene expression changed by the blue light induction. At the same time, it was found that the expression with blue light was higher than the control, indicating that external blue light stimulation might increase the expression of Gl14-3-3 gene. It was suggested that the 14-3-3 protein induced by blue light may be related to mycelial differentiation, fruiting body formation and secondary metabolites synthesis of *G. lucidum*.

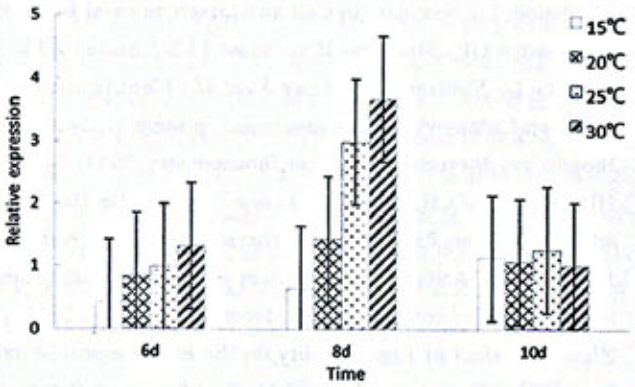

Fig. 7 The relative expression of Gl14-3-3 genewith different temperatures

It has been reported that the expression of 14-3-3 proteinin *Hypsizigus marmoreus* increased during hypothermia treatment[12]. However the expression of Gl14-3-3 gene decreased after low temperature treatment. The two fungi show different performances. *Hypsizigus marmoreus* is a kind of low-temperature fruiting edible fungus and suitable to forming the fruiting body at 12-14℃, while the temperature of forming the fruiting body of *G. lucidum* is higher, 18-32℃. The optimum temperature for the growth of the two fungi are very different. So the 14-3-3 protein reacts differently, reflecting the response to the environment.

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