Abstract

Ganoderma lucidum is a mushroom with traditional medicinal properties that has been widely used in China and other countries in Eastern Asia. Ganoderic acids (GA) produced by G. lucidum exhibit important pharmacological activities. Previous studies have demonstrated that methyl jasmonate (MeJA) is a potent inducer of GA biosynthesis and the expression of genes involved in the GA biosynthesis pathway in G. lucidum. To further explore the mechanism of GA biosynthesis, cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) was used to identify genes that are differentially expressed in response to MeJA. Using 64 primer combinations, over 3910 transcriptionally derived fragments (TDFs) were obtained. Reliable sequence data were obtained for 390 of 458 selected TDFs. Ninety of these TDFs were annotated with known functions through BLASTX searching the GenBank database, and 12 annotated TDFs were assigned into secondary metabolic pathways by searching the KEGGPATHWAY database. Twenty-five TDFs were selected for qRT-PCR analysis to confirm the expression patterns observed with cDNA-AFLP. The qRT-PCR results were consistent with the altered patterns of gene expression revealed by the cDNA-AFLP technique. Additionally, the transcript levels of 10 genes were measured at the mycelium, primordia, and fruiting body developmental stages of G. lucidum. The greatest expression levels were reached during primordia for all of the genes except cytochrome b2 reached its highest expression level in the mycelium stage. This study not only identifies new candidate genes involved in the regulation of GA biosynthesis but also provides further insight into MeJA-induced gene expression and secondary metabolic response in G. lucidum.

Introduction

Medicinal mushrooms are viewed as a rich source of therapeutically useful biologically active agents. There are approximately 700 species of higher basidiomycetes that have been found to possess significant pharmacological activities [1]. For several thousand years, Ganoderma lucidum (Ling-Zhi in Chinese and Reishi in Japanese) has been widely used in Asia as a home remedy to treat minor disorders and promote vitality and longevity [2]. Numerous studies have revealed that the primary active ingredients of G. lucidum are polysaccharides and the secondary metabolites ganoderic acids (GAs) [3,4]. Most GAs have important medicinal value, such as the regulation of osteoclast genesis [5], the inhibition of cholesterol synthesis [3] and tumor growth [6], and protection of the liver [7]. However, despite the important pharmacological potential of GAs, low GA yield from both field cultivation and fermentation limits its wide-spread use.

Many attempts have been made to increase GA biosynthesis. Those works can be divided into two branches. Most reports focus on the environmental conditions during fermentation. The optimal medium (carbon source, nitrogen source, mineral source, and initial pH) was elucidated by an orthogonal design study that tested one factor at a time [8]. By studying the effect of the fed-batch fermentation process (pH-shift and dissolved oxygen tension-shift) on the GA content, strategies were identified that resulted in a significant synergistic enhancement of GA accumulation [9]. Recently, the use of an inducer to enhance the activity components in fungi fermentation has drawn great interest [10,11]. For GA production, methyl jasmonate, phenobarbitral and H2O2 were added to culture medium to increase the GA content [12–14]. However, due to the unclear mechanism of ganoderic acid biosynthesis, determining the optimal fermentation conditions and screening an effective inducer to produce maximum quantities of GA are still a trial-and-error process.

Isotopic tracer experiments have demonstrated that GA, a type of terpenoid, is synthesized via the mevalonate pathway [15,16]. The genes that encode the proteins involved in the GA biosynthesis pathway have been cloned and characterized, and the regulation of the expression levels of these genes has been investigated under different environmental conditions to deter-
mine the relationship between GA biosynthesis and the expression of these genes [17–20]. Recent studies have demonstrated that the over-expression of these biosynthetic genes results in an enhanced accumulation of GA in G. lucidum [21–23]. Although these reports indicated that increased GA biosynthesis may result from the up-regulation of GA biosynthesis genes, how specific environmental conditions induce GA biosynthesis through the GA biosynthetic pathway remains unclear. Therefore, research on the GA biosynthesis mechanism has concentrated on the mevalonate pathway.

MeJA is a ubiquitous small signaling molecule in the plant kingdom. Environmental stresses, such as wounding or pathogen attack, can trigger MeJA production [24,25]. In plants, MeJA induces stomatal closure, monoterpenoid indole alkaloids and isoprenoid biosynthesis, and defense response pathways by activating reactive oxygen species, the MAPK signal pathway, or the calcium-dependent protein kinase signal pathway [25–28]. In fungi, MeJA is involved in the modulation of Cryptococcus laurentii and Penicillium expansum growth [29] and the regulation of Aflatoxin B1 biosynthesis by Aspergillus parasiticus [30,31]. The same phenomenon is observed in G. lucidum; when different concentrations of MeJA were added to the culture, the GA contents were improved [12]. Moreover, the transcript levels of the genes hmg, fps, and sgs in the GA biosynthesis pathway were up-regulated in response to MeJA. However, the signaling pathways initiated by MeJA to regulate GA biosynthesis and gene expression remain unknown.

In this study, differentially expressed transcripts were screened in the MeJA-treated mycelium using cDNA-AFLP to gain insights into the regulatory mechanisms of GA biosynthesis in response to MeJA. The differentially expressed transcripts were sequenced and classified, and their expression patterns were analyzed. For some of the regulated genes, quantitative real-time polymerase chain reaction (qRT-PCR) was used to confirm the expression patterns observed with cDNA-AFLP. In addition, the transcript levels of some of the candidate genes were investigated at various developmental stages of G. lucidum.

Results

Isolation of Differentially Expressed Genes

To select a suitable restriction enzyme combination for cDNA-AFLP analysis of G. lucidum, several enzyme combinations were tested; the combination of EcoRI and MseI produced an acceptable range of fragment sizes (Figure 1).

A total of 64 primer combinations was used to selectively amplify the expressed genes. Differentially expressed transcript-derived fragments were extracted from the gel and used as templates for re-amplification by PCR. The cDNA-AFLP fragments were highly reproducible, as evidenced by the similar band intensities observed in the three biological replications. All of the visible TDFs between 150 and 800 bp were counted. Of the total 3910 transcript-derived fragments obtained using cDNA-AFLP with 64 primer pairs, 919 (23.5%) displayed altered expression patterns after MeJA induction; 703 were up-regulated, and 216 were down-regulated. Reliable sequences were obtained for 390 TDFs out of 458 TDFs selected for further analysis. Sequence data from this article have been deposited in GenBank, Accession Numbers: JZ163375-JZ163764. According to the genomic sequence of G. lucidum [32,33], the distributions of 390 TDFs were analyzed as shown in Figure S1. Because our knowledge of gene functions in G. lucidum is relatively limited, only 90 of the sequenced genes were associated with known functions, as determined by BLAST searching the GenBank database (Table 1 and Table 2). The sites of known functional TDFs on chromosomes were analyzed as shown in Table 2 and Figure S2. Several differentially expressed genes showed homology to genes encoding transcription factors and genes involved in metabolism, gene regulation, signal transduction, stress defense, protein trafficking and protein degradation (Table 2).

Gene Sequence Analysis

The annotation approach was based on sequence similarity searches in the GenBank database. The 390 TDFs were subjected to a BLASTX search against the NCBI non-redundant protein database using the default parameters. The results revealed that 241 TDFs (61.8%) had significant sequence similarities to known proteins (eValue<10^-5); 90 TDFs (23.08%) had significant sequence similarity to classified proteins, 151 TDFs (38.72%) had sequence similarity to unclassified proteins; and the remaining 149 TDFs (38.21%) failed to match any proteins in the database. It was noted that the information about the genomes or transcriptomes of this species was needed in-depth analysis. Of the 90 TDFs, 45.6% were homologous to Coprinopsis cinere, 11.1% were homologous to Laccaria bicolor and 7.8% were homologous to Postia placenta.

Gene ontology (GO) assignments describe gene products in terms of their associated molecular functions, biological processes and cellular components. Blast2GO (B2G) is a bioinformatic tool for GO-based DNA or protein sequence annotation [34]. The 90 TDFs were submitted to Blast2GO, and 75 were successfully annotated (Table S1). Figure 2A shows the percentages of differentially expressed genes in the 90 known functional sequences assigned to various functional categories. Of these, 40.0% of the annotations were related to ‘metabolism/energy’, 17.0% were related to ‘protein synthesis/fate’, and 16.0% were related to ‘transcription’. Figure 2B shows that the percentages of different functional categories of up- and down-regulated sequences in the 90 known functional sequences were significantly different (P<0.05). In the metabolism/energy group and the protein synthesis/fate group, the percentages of up-regulated genes (28.9% and 13.3%, respectively) were increased compared with the percentages of down-regulated genes (11.1% and 3.3%, respectively). In the transcription term and the signal transduction term, the percentages of up-regulated genes (7.8% and 5.6%, respectively) were equal to the percentages of down-regulated genes (7.8% and 5.6%, respectively). Interestingly, all of the differentially expressed genes were up-regulated in the defense/cell organization group (4.4%). KEGG provides a reference knowledge base for linking genomes to life through the process of PATHWAY mapping. In this study, the 90 annotated TDFs were blasted against the KEGG database. Thirty of these TDFs were highly homologous to some protein, and 21 were assigned to the metabolic pathways in the database (Table S2). Interestingly, 12 TDFs were assigned to secondary metabolic pathways, specifically, the biosynthesis of terpenes, terpenoids and steroids.

Quantitative RT-PCR Analysis of MeJA-induced Differentially Expressed TDFs in G. lucidum

Co-expression analysis, which is based on the premise that a set of genes involved in a biological process is co-expressed under given conditions, has been successfully used to identify novel genes involved in secondary metabolism [35]. To verify the correlation between the expression of differentially expressed TDFs and MeJA induction, quantitative RT-PCR analysis was performed for 25 TDFs, which involved in metabolism [glucosidase I (gls), glutathione-dependent formaldehyde dehydrogenase (gfd), fumarase...
(fum), NAD-dependent deacetylase (ndd), pyruvate carboxylase (pco), pyruvate kinase (pyr), ERG27-3-keto sterol reductase (ksr), aryl-alcohol oxidase (aao), catalase (cat), cytochrome b2 (cyt) and acetyl-CoA acetyltransferase (aact)), gene regulation (nucleotide binding protein (nbp), histone deacetylase (hd), pre-mRNA splicing factor (prp) and IMP-specific 5′-nucleotidase 1 (nuc)), signal transduction (cAMP-dependent protein kinase (apk), CMGC/MAPK/JNK protein kinase (mapk), small monomeric GTPase (rho), histidine kinase (hk) and protein kinase activator (mob)), cell organization (cell division control protein (cdc)) and trafficking (vacuolar membrane protein (vmp), vacuole protein (vac), calcium transporting ATPase (cal) and glycerol uptake facilitator (guf)). In Figure 3, nbp (TDF009), cal (TDF375), hd (TDF293), gls (TDF080), apk (TDF040), gfd (TDF291), guf (TDF078), prp (TDF042), ndd (TDF015), mapk (TDF013), and rho (TDF165) were down-regulated in response to MeJA treatment, whereas others were up-regulated. For hk (TDF051), mob (TDF052), nuc (TDF058), pyr (TDF323), pco (TDF243), ksr (TDF256), and vmp (TDF264), the highest levels of transcripts were observed with 50 µM MeJA. For aao (TDF096), cdc (TDF122), cat (TDF129), fum (TDF195), vac (TDF360), cyt (TDF047), and aact (TDF113), the highest levels of transcripts were observed with 200 µM MeJA. The qRT-PCR results are

Figure 1. cDNA-AFLP analysis of transcripts in response to MeJA treatment in *G. lucidum*. cDNA-AFLP silver-stained polyacrylamide gels with 9 primer combinations (PC) amplifying differentially expressed genes in *G. lucidum* treated with 0, 50 and 200 µM MeJA, respectively. The combinations of primers used are indicated according to the codes reported in Table S4. The molecular weight marker sizes are indicated on both sides. Arrows indicate some of the differentially expressed transcript-derived fragments.

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consist with the altered expression patterns observed for these 23 genes using cDNA-AFLP (Figure S3).

Variations in Gene Expression at Developmental Stages of G. lucidum

All of TDFs from MeJA-induced library were searched from mycelium or fruiting body EST library reported by Chen et al., 2012 [32] (Figure 4A and Table S3). In the three EST libraries, there are 260 genes accounted for the majority, including basic metabolism and signal transduction genes. There are 30 genes appeared in both mycelium library and MeJA-induced library, most of which are of unknown function genes. Forty-nine genes appeared in both the fruiting EST library and MeJA-induced library, including ERG27-3-keto sterol reductase and cytochrome P450. Fifty genes only exist in MeJA-induced library, indicating that these genes inducing conditions expressed in methyl jasmonate, and may not be expressed or low expressed in mycelium and fruiting bodies, such as pre-mRNA splicing factor and catalase.

Varying amounts of GA are observed in different developmental stages in G. lucidum. A recent study reported that the GA level is highest during the primordium and fruiting body stages [32]. To further study the relationship between the differentially expressed genes and GA biosynthesis, the transcription levels of 10 genes were examined during the mycelium, primordium, and fruiting body developmental stages in G. lucidum (Figure 4B). Expression levels were the highest during primordium for TDF040 (apk, cAMP-dependent protein kinase), TDF096 (aoa, aroyl-alcohol oxidase), TDF052 (mob, protein kinase activator), TDF256 (ksr, ERG27-3-keto sterol reductase), TDF051 (hk, histidine kinase), TDF103 (mapk, CMGC/MAPK/JNK protein kinase), and TDF165 (rho, small monomeric GTPase). For TDF264 (vmp, vacuolar membrane protein) and TDF009 (nrbp, nucleotide binding protein), expression levels were the highest during both the primordium and the fruiting body stages. Only TDF047 (ct, cytochrome b2) showed a maximum expression level during the mycelium stage.

Discussion

The biosynthesis of many secondary metabolites is modulated by environmental conditions [36]. Few data are available to elucidate the regulatory mechanisms of the secondary metabolite biosynthesis in response to environmental factors in fungi due to the complex regulatory network and regulatory gene interactions involved [36–38]. The same challenge exists for understanding the mechanisms governing the regulation of GA biosynthesis [39,40]. Despite significant research efforts to understand the influence of environmental factors on the GA biosynthesis [39], the regulatory networks by which environmental factors regulate GA biosynthesis remain unclear. Recent studies on genetically modified GA biosynthesis have focused on the genes restricted to the mevalonate pathway [21,22]. Our previous study demonstrated that methyl jasmonate can significantly increase both the amount of GA and the transcription levels of known genes in the GA biosynthesis pathway [12]. Additional unknown genes may be involved in the regulatory network of GA biosynthesis. Therefore, the screening of differentially expressed genes during MeJA induction may not only identify novel candidate target genes involved in the regulation of GA biosynthesis but may also provide a new perspective for understanding the regulation of GA biosynthesis (Figure 5).

Among the MeJA up-regulated genes were the acetyl-CoA acetyltransferase gene (TDF113), several members of the cytochrome family (TDF019, TDF047, TDF160, TDF313, TDF322, TDF355, and TDF364), and, most notably, cytochrome P450s (CYPs) (Table 2 and Figure 3). In addition, TDF256, which encodes a 3-keto sterol reductase (ksr), was shown to be up-regulated by MeJA induction with both cDNA-AFLP and real-time PCR (Table 2 and Figure 3). In Saccharomyces cerevisiae, ksr (Erg27p) is required for oxidosqualene cyclase (Erg7p) activity [41], which converts oxidosqualene to lanosterol. Those results suggest that the genes in the mevalonate pathway are up-regulated in response to MeJA induction (Figure 5). This result is consistent with those of previous studies [12,13] and demonstrates that the genes encoding key enzymes in the mevalonate pathway play an important role in GA biosynthesis. Although not all of the differentially expressed genes in the mevalonate pathway have been detected, the cDNA-AFLP approach is effective for screening differentially expressed genes during MeJA induction.

These results indicate that MeJA induction modulates not only GA biosynthesis-related genes but also related genes in other metabolic pathways, such as glycerol metabolism, pyruvate metabolism, lactate metabolism, sphingolipid metabolism etc. Pyruvate is a precursor of the methylerythritol 4-phosphate (MEP) pathway, and a pyruvate decarboxylase catalyzes the formation of pyruvate decarboxylase catalyzes the formation of pyruvate carboxylase (Figure 3). This result is consistent with those of previous studies [12,13] and demonstrates that the genes encoding key enzymes in the mevalonate pathway play an important role in GA biosynthesis. Although not all of the differentially expressed genes in the mevalonate pathway have been detected, the cDNA-AFLP approach is effective for screening differentially expressed genes during MeJA induction.

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| No. | TDF | Size | Metabolism/energy | Homologue | Max Score | Max ident* | E value | Expressionb | Chromosomes Sitec |
|-----|-----|------|------------------|-----------|----------|-----------|---------|-------------|------------------|
| 1   | TDF006 | 271 | Glucosidase I (Coprinopsis cinerea) | 140 | 71% | 6.0E-32 U | No hits found |
| 2   | TDF015 | 468 | NAD-dependent deacetylase (Puccinia graminis) | 120 | 42% | 9.0E-26 D | Chr6 |
| 3   | TDF070 | 267 | Ceramidase (Coprinopsis cinerea) | 243 | 77% | 9.0E-63 U | Chr13 |
| 4   | TDF080 | 365 | 1,3-beta-glucan synthase (Laccaria bicolor) | 171 | 80% | 3.0E-41 D | Chr11 |
| 5   | TDF096 | 275 | Aryl-alcohol oxidase (Coprinopsis cinerea) | 95.9 | 70% | 2.0E-18 U | No hits found |
| 6   | TDF300 | 375 | Phosphoglycerate kinase (Coprinopsis cinerea) | 365 | 79% | 2.0E-123 D | No hits found |
| 7   | TDF138 | 677 | Anthranilate synthase (Coprinopsis scobicola) | 194 | 79% | 6.0E-59 D | Chr3 |
| 8   | TDF142 | 494 | Saccharopine dehydrogenase (Coprinopsis cinerea) | 194 | 79% | 6.0E-57 U | Chr11 |
| 9   | TDF143 | 461 | 2-methylcitrate dehydratase (Coprinopsis cinerea) | 192 | 74% | 7.0E-57 U | Chr11 |
| 10  | TDF047 | 612 | Cytochrome b2 (Coprinopsis cinerea) | 180 | 66% | 1.0E-43 U | Chr3 |
| 11  | TDF099 | 262 | Acetylactylate synthase (Neosartorya fischeri) | 86.3 | 55% | 1.0E-15 U | Chr7 |
| 12  | TDF375 | 194 | Calcium transporting ATPase (Coprinopsis cinerea) | 63.2 | 100% | 6.0E-15 D | Chr13 |
| 13  | TDF153 | 478 | Aspartate ammonia lyase (Coprinopsis cinerea) | 272 | 84% | 3.0E-88 U | Chr1 |
| 14  | TDF376 | 538 | Lipase/esterase (Coprinopsis cinerea) | 155 | 43% | 1.0E-40 D | Chr1 |
| 15  | TDF113 | 600 | Acetyl-CoA acetyltransferase (Coprinopsis cinerea) | 302 | 74% | 2.0E-80 U | Chr1 |
| 16  | TDF160 | 718 | Cytochrome P450 (Postia placenta) | 239 | 55% | 7.0E-74 U | Chr11 |
| 17  | TDF161 | 511 | Glycoside hydrolase family 31 protein (Serpula lacrymans) | 225 | 62% | 3.0E-67 U | Chr3 |
| 18  | TDF381 | 309 | Flavin-containing monooxygenase (Aspergillus niger) | 104 | 49% | 3.0E-24 U | Chr12 |
| 19  | TDF313 | 287 | Cytochrome-b5 reductase (Coprinopsis cinerea) | 99.4 | 78% | 6.0E-47 U | GaLu96scf_50 |
| 20  | TDF314 | 327 | Hexokinase (Coprinopsis cinerea) | 151 | 81% | 1.0E-41 U | Chr11 |
| 21  | TDF019 | 265 | Cytochrome b5 (Phanerochaete chrysosporium) | 123 | 76% | 2.0E-34 U | Chr1 |
| 22  | TDF353 | 278 | Heparinsacu II/III family protein (Coprinopsis cinerea) | 128 | 56% | 6.0E-32 D | Chr3 |
| 23  | TDF355 | 219 | Cytochrome P450 (Postia placenta) | 147 | 65% | 5.0E-40 U | Chr1 |
| 24  | TDF243 | 238 | Pyruvate carboxylase (Laccaria bicolor) | 142 | 84% | 1.0E-38 U | Chr6 |
| 25  | TDF195 | 478 | Heparinase II/III family protein (Coprinopsis cinerea) | 128 | 56% | 6.0E-32 D | Chr3 |
| 26  | TDF136 | 282 | Cytochrome P450 like TBP (Scheffersomyces stipitis) | 269 | 82% | 2.0E-47 U | Chr3 |
| 27  | TDF307 | 239 | Fumarase (Scheffersomyces stipitis) | 117 | 73% | 2.0E-29 U | Chr11 |
| 28  | TDF223 | 170 | Glycoside hydrolase family 3 protein (Serpula lacrymans) | 75.1 | 61% | 2.0E-15 U | Chr4 |
| 29  | TDF338 | 209 | ATP synthase subunit gamma (Coprinopsis cinerea) | 85.1 | 90% | 2.0E-18 U | Chr11 |

**Transcription**

| No. | TDF | Size | Homologue | Max Score | Max ident* | E value | Expressionb |
|-----|-----|------|-----------|----------|-----------|---------|-------------|
| 37  | TDF009 | 336 | Nucleotide binding protein, putative (Candida dubliniensis) | 103 | 50% | 6.0E-21 D | Chr3 |
| 38  | TDF020 | 330 | Transcription factor (Sterum hirsutum) | 211 | 90% | 3.0E-62 U | Chr1 |
| 39  | TDF042 | 271 | Pre-mRNA splicing factor prp1 (Coprinopsis cinerea) | 113 | 63% | 1.0E-23 D | Chr7 |
| 40  | TDF058 | 551 | IMP-specific 5'-nucleotidase 1 (Uncinocarpus reessii) | 221 | 59% | 5.0E-56 U | Chr3 |
| 41  | TDF081 | 316 | Epsilon DNA polymerase (Coprinopsis cinerea) | 156 | 71% | 9.0E-37 D | Chr1 |
| 42  | TDF318 | 309 | Eukaryotic translation initiation factor 6 (Postia placenta) | 156 | 95% | 1.0E-46 U | Chr8 |
| 43  | TDF094 | 379 | DNA-directed RNA polymerase II subunit (Coprinopsis cinerea) | 206 | 91% | 1.0E-51 D | Chr6 |
| 44  | TDF114 | 466 | Translation initiation factor 3 subunit 3 (Coprinopsis cinerea) | 171 | 71% | 5.0E-41 U | Chr3 |
| 45  | TDF050 | 398 | Chrom domain protein MRG15 (Piriformospora indica) | 174 | 47% | 1.0E-50 D | Chr12 |
| 46  | TDF049 | 374 | Translation elongation factor 1a (Schizopyllum commune) | 266 | 94% | 6.0E-85 U | Chr3 |
| 47  | TDF370 | 233 | rRNA intron-encoded homing endonuclease (Medicago trunculata) | 137 | 49% | 1.0E-34 D | Chr5 |

**Table 2. Transcript derived fragments (TDFs) from G. lucidum with homologies to other known protein.**
### Table 2. Cont.

| No. | TDF   | Size | Homologue*                                                                 | Max Score | Max ident | E value | Expression | Chromosomes Site |
|-----|-------|------|---------------------------------------------------------------------------|-----------|-----------|---------|------------|------------------|
| 48  | TDF341 303 | RWD domain-containing protein (Laccaria bicolor) | 141       | 57%       | 8.00E-40 | D        | No hits found |
| 49  | TDF156 340 | argonaute-like protein (Laccaria bicolor) | 140       | 66%       | 1.00E-36 | D        | Chr11           |
| 50  | TDF390 320 | transcription factor (Stereum hirsutum) | 204       | 90%       | 8.00E-60 | U        | Chr1            |

### Protein synthesis/fate

| No. | TDF   | Size | Homologue*                                                                 | Max Score | Max ident | E value | Expression | Chromosomes Site |
|-----|-------|------|---------------------------------------------------------------------------|-----------|-----------|---------|------------|------------------|
| 51  | TDF297 312 | peptidylprolyl isomerase (Datisca glomerata) | 186       | 82%       | 4.00E-59 | U        | Chr9            |
| 52  | TDF136 444 | ubiquitin-protein ligase (Coprinopsis cinerea) | 208       | 68%       | 5.00E-61 | U        | Chr4            |
| 53  | TDF299 304 | histone H2B (Coprinopsis cinerea) | 176       | 100%      | 2.00E-54 | U        | Chr2            |
| 54  | TDF145 561 | 60S ribosomal protein L10 (Postia placenta) | 333       | 95%       | 2.00E-115 | U        | Chr10           |
| 55  | TDF303 439 | profilin (Laccaria bicolor) | 186       | 74%       | 1.00E-58 | U        | Chr9            |
| 56  | TDF151 501 | SNARE protein SEDS | 192       | 78%       | 2.00E-58 | U        | Chr3            |
| 57  | TDF164 405 | mitochondrial endopeptidase (Serpula lacrymans) | 198       | 66%       | 2.00E-58 | U        | Chr7            |
| 58  | TDF312 272 | histone H4 (Coprinopsis cinerea) | 160       | 100%      | 3.00E-50 | U        | Chr6            |
| 59  | TDF383 181 | signal peptidase 21 kDa subunit (Coprinopsis cinerea) | 113       | 91%       | 4.00E-31 | U        | Chr11           |
| 60  | TDF347 333 | Ubiquitin (Camponotus floridanus) | 246       | 98%       | 2.00E-82 | U        | Chr3            |
| 61  | TDF385 286 | mitochondrial 50S ribosomal protein L5 (Postia placenta) | 84.7      | 84%       | 1.00E-18 | D        | Chr7            |

### Signal transduction

| No. | TDF   | Size | Homologue*                                                                 | Max Score | Max ident | E value | Expression | Chromosomes Site |
|-----|-------|------|---------------------------------------------------------------------------|-----------|-----------|---------|------------|------------------|
| 66  | TDF013 297 | CMGC/MAPK/JNK protein kinase (Coprinopsis cinerea) | 104       | 58%       | 4.00E-21 | D        | Chr4            |
| 67  | TDF193 736 | Rho2 GTP binding protein (Ustilago maydis) | 127       | 88%       | 1.00E-50 | D        | Chr2            |
| 68  | TDF040 299 | CaM-dependent protein kinase Akt (Coprinopsis cinerea) | 90.5      | 49%       | 7.00E-17 | D        | Chr1            |
| 69  | TDF051 669 | histidine kinase (Schizosaccharomyces pombe) | 116       | 62%       | 3.00E-24 | U        | Chr6            |
| 70  | TDF052 547 | protein kinase activator Mob2 (Coprinopsis cinerea) | 203       | 78%       | 8.00E-51 | U        | Chr7            |
| 71  | TDF304 299 | serine/threonine kinase receptor associated protein (Coprinopsis cinerea) | 84%       | 84%       | 3.00E-60 | U        | No hits found |
| 72  | TDF158 580 | CMGC/GSK protein kinase (Coprinopsis cinerea) | 159       | 81%       | 6.00E-45 | U        | Chr4            |
| 73  | TDF165 675 | rho small monomeric GTPase (Coprinopsis cinerea) | 268       | 74%       | 6.00E-89 | D        | Chr2            |
| 74  | TDF008 848 | Ras2 (Cryptococcus neoformans) | 89       | 69%       | 8.00E-35 | D        | Chr8            |
| 75  | TDF333 394 | signal recognition particle binding protein (Coprinopsis cinerea) | 187       | 90%       | 1.00E-53 | U        | No hits found |

### Transport facilitation

| No. | TDF   | Size | Homologue*                                                                 | Max Score | Max ident | E value | Expression | Chromosomes Site |
|-----|-------|------|---------------------------------------------------------------------------|-----------|-----------|---------|------------|------------------|
| 76  | TDF078 444 | glycerol uptake facilitator (Talaromyces stipitatus) | 105       | 45%       | 2.00E-21 | D        | Chr5            |
| 77  | TDF083 441 | copper chaperone TahA (Trametes versicolor) | 117       | 83%       | 8.00E-25 | U        | Chr8            |
| 78  | TDF159 548 | vacuolar sorting protein (Ajellomyces capsulatus) | 153       | 43%       | 2.00E-43 | D        | Chr3            |
| 79  | TDF187 490 | oligopeptide transporter (Laccaria bicolor) | 266       | 76%       | 3.00E-83 | U        | Chr10           |
| 80  | TDF342 350 | inorganic phosphate transporter (Laccaria bicolor) | 99       | 76%       | 2.00E-22 | D        | Chr8            |
| 81  | TDF048 218 | nuclear transport factor 2 (Coprinopsis cinerea) | 174       | 80%       | 5.00E-54 | D        | Chr10           |
| 82  | TDF360 315 | vacuole protein (Cryptococcus neoformans) | 96.3      | 64%       | 1.00E-23 | U        | Chr3            |
| 83  | TDF327 167 | COPII-coated vesicle protein (Coprinopsis cinerea) | 85.9      | 85%       | 3.00E-20 | U        | Chr1            |
| 84  | TDF384 259 | NIFU-like protein c (Coprinopsis cinerea) | 126       | 74%       | 3.00E-34 | D        | Chr3            |
| 85  | TDF316 294 | t-complex protein 1 (Coprinopsis cinerea) | 162       | 73%       | 8.00E-46 | U        | Chr1            |
| 86  | TDF264 521 | vacuolar membrane protein (Cryptococcus gattii) | 97.8      | 73%       | 6.00E-28 | U        | Chr4            |
membrane protein up-regulated under MeJA treatment and in fruiting body development. The consistent result indicated these five genes may have important roles on the GA biosynthesis. However, there are four genes down-regulated under MeJA treatment and up-regulated in fruiting body development (cAMP-dependent protein kinase, CMGC/MAPK/JNK protein kinase, small monomeric GTPase and nucleotide binding protein) (Figure 3 and Figure 4). Although both MeJA induction and fruiting body stage increased GA content, the regulation mechanism may be difference. These genes may have a variety of physiological functions, especially the regulation of fungal development in development process [42,43]. Therefore, these four genes probably regulated not only GA biosynthesis in the fruiting body stage, but also the fruiting body development of *Ganoderma lucidum*.

Some signaling factors have been reported to regulate both cell developmental processes and secondary metabolite biosynthesis in filamentous fungi [37]. For example, the small monomeric GTPase rho played an essential role in controlling genes involved in cell polarity, H₂O₂ generation, asexual sporulation, and mycotoxin production [42,44,45]. The rho-GTPase pathway was associated with cholesterol biosynthesis [46]. In the cDNA-AFLP library, the *G. lucidum* TDF165 gene encodes a protein with sequence similarity to rho (Table 2). The qRT-PCR results confirmed that the rho gene is down-regulated in response to MeJA (Figure 3). Furthermore, the down-regulation of the rho expression level through rho gene silencing can increase the GA content (data not shown). These results suggest that the rho small GTPase pathway represses GA biosynthesis in *G. lucidum*.

A mitogen-activated protein kinase (MAPK) gene (TDF013) was also isolated and characterized from the *G. lucidum* library. The transcription level of the MAPK gene in response to MeJA was 0.20±0.01-fold that of the control. As one of components of the MAPK cascade, a critical signal transduction pathway in eukaryotic organisms, MAPK is essential for regulating growth, differentiation processes and secondary metabolism [47,48]. Recently, it was reported that the MAPK cascade played an important role in regulating sterigmatocystin biosynthesis [43]. In *G. lucidum*, H₂O₂ induced phosphorylation of the proteins Hog-1 and Fus3, which are homologs of the mammalian MAPKs p38 and ERK [49]. In contrast, the mammalian JNK homolog in BCRC 36111 was not detected using a JNK-specific antibody [50]. In this study, the CMGC/MAPK/JNK MAPK is down-regulated after MeJA induction, suggesting that it may

**Table 2. Cont.**

| No. | TDF   | Size | Homologue* | Max Score | Max ident | E value | Expression | Chromosomes |
|-----|-------|------|------------|-----------|-----------|---------|------------|-------------|
| 87  | TDF122| 674  | cell division control protein 23 (Coprinopsis cinerea) | 236       | 65%       | 2.00E-60 | U          | Chr5        |
| 88  | TDF176| 346  | hsp70-like protein (Coprinopsis cinerea) | 91.7      | 71%       | 4.00E-20 | U          | Chr10       |
| 89  | TDF179| 280  | microtubule associated protein (Coprinopsis cinerea) | 99        | 75%       | 4.00E-23 | U          | No hits found |
| 90  | TDF129| 448  | catalase (Trametes versicolor) | 216       | 66%       | 1.00E-63 | U          | Chr3        |

*Based on highest BLASTX match with an E value lower than 1e-04.

U, up-regulation; D, down-regulation.

The distribution of TDFs on chromosomes in *G. lucidum* genome.

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**Figure 2. Percentages of 90 known functional TDFs with functional categories.** A. The functional classification of transcript-derived fragments (90 TDFs) from *G. lucidum* in response to MeJA. The percentages of differentially expressed genes in the 90 known functional sequences assigned to various functional categories. B. Ninety TDFs in biological function categories showing differential expression patterns in *G. lucidum*. The percentages of different functional categories of up- and down-regulated sequences in the 90 known functional sequences were significantly different (P<0.05).

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play a negative regulatory role in the MeJA signaling pathway (Figure 5).

The sequences of the TDFs that generated significant matches to sequence databases were most commonly genes involved in stress response and cell organization. Two of these TDFs, TDF122, which encodes cell division control protein, and TDF176, which encodes an hsp70-like protein, may also be involved in cell repair and protection against defense responses. TDF 145 and TDF336, which are derived from putative 60S ribosomal protein genes, have also been implicated in responses to oxidative stress, in addition to protein translation for improving carotenoid biosynthesis [51]. Other TDFs, such as *G. lucidum* TDF129 (similar to a catalase), are clearly involved in oxidative stress defense [52]. Previous studies reported that H2O2 increases GA production [14]. Catalase might be involved in prompt neutralization of H2O2. The up-regulation of TDF129, a catalase-homologous gene in response to MeJA, indicated that the burst of

Figure 3. qRT-PCR analysis of 25 selected TDFs in *G. lucidum*. Expression of 25 selected genes treated with 0, 50 and 200 μM MeJA, respectively. aao (TDF096) aryl-alcohol oxidase, nbp (TDF009) nucleotide binding protein, cdc (TDF122) cell division control protein, cal (TDF375) calcium transporting ATPase, cat (TDF129) catalase, vmp (TDF264) vacuolar membrane protein, hd (TDF293) histone deacetylase, gls (TDF080) glucosidase I, hk (TDF051) histidine kinase, fum (TDF195) fumarase, ksr (TDF256) ERG27-3-keto sterol reductase, apk (TDF040) cAMP-dependent protein kinase, nuc (TDF058) IMP-specific 5’-nucleotidase I, gfd (TDF291) glutathione-dependent formaldehyde dehydrogenase, ppyr (TDF323) pyruvate kinase, mob (TDF052) protein kinase activator, pec (TDF243) pyruvate carboxylase, guf (TDF078) glycerol uptake facilitator, prp (TDF042) pre-mRNA splicing factor, vac (TDF360) vacuole protein, aact (TDF113) acetyl-CoA acetyltransferase, ndd (TDF015) NAD-dependent deacetylase, mapk (TDF013) CMGC/MAPK/JNK protein kinase, rho (TDF165) small monomeric GTPase, cyt (TDF047) cytochrome b2. All samples were examined in triplicate. For all genes represented in this figure, the P value was <0.05 or 0.01 (*p<0.05 and **p<0.01).

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reactive oxygen species (ROS) triggered by MeJA was most likely involved in GA biosynthesis (Figure 5).

Vacuoles and vesicles are known to sequester secondary metabolites to protect host cells from self-toxicity [53]. Enzymes involved in secondary metabolism, including those for the biosynthesis of cyclosporin, penicillin, and aflatoxin in fungi, are often found in vesicles and vacuoles, [54–56]. In *Aspergillus parasiticus*, two enzymatic steps in aflatoxin biosynthesis are completed in vesicles, and these organelles also participate in the compartmentalization and export of the end product, aflatoxin [57]. A vacuole protein gene (TDF360), a vacuolar membrane protein (TDF264) and a COPII-coated vesicle protein gene (TDF327) were also isolated from the *G. lucidum* library (Table 2).

The transcription level of the *vmp* gene in response to MeJA induction was 3.10±0.12-fold that of the control (Figure 3).

In conclusion, cDNA-AFLP screening has revealed a number of MeJA-responsive genes in *G. lucidum*. Of the 390 successfully sequenced TDFs, 300 unknown or hypothetical proteins require

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**Figure 4.** The expression of TDF genes in different development stages. A. Venn diagrams depicting the genes expressed across MeJA-induction and the different developmental stages. Data are derived from Table S3. B. The transcript levels of TDF genes under the developmental stages of *G. lucidum*. The X axis shows the abbreviation of genes. The full name of each gene is in the Figure 3 legends. All samples were examined in triplicate. For all genes represented in this figure, the P value was <0.01. doi:10.1371/journal.pone.0065027.g004

**Figure 5.** Schematic pathway predicting the role of MeJA induced genes in *G. lucidum*. Integrated pathway map shows the role of MeJA-induced genes involved in GA biosynthesis, primary metabolism, signaling regulation and transcriptional regulation. Dashed lines indicate the probable pathway involved in GA biosynthesis. Solid lines indicate the result supported by cDNA-AFLP and real time PCR. The ESTs from cDNA-AFLP results are indicated in stars. doi:10.1371/journal.pone.0065027.g005
The MeJA induction leads to altered metabolism/energy of genes and the Ca$^{2+}$ previous report found that the calcineurin-signal transduction pyruvate metabolism, calcium transporting ATPases etc. In other secondary metabolism, such as glycerol metabolism, sterilized using a 0.2-
concentration was 2
in the GA biosynthesis pathway [12]. The MeJA induction leads to altered metabolism/energy of G. lucidum, which involves changes in primary metabolism and other secondary metabolism, such as glycerol metabolism, pyruvate metabolism, calcium transporting ATPases etc. In previous report found that the calcineurin-signal transduction was significant to GA biosynthesis [58]. The GA biosynthetic genes and the Ca$^{2+}$ sensor were up-regulated with calcium addition. The changes of CMGC/GSK protein kinase, histidine kinase, serine/threonine kinase receptor associated protein, cAMP-dependent protein kinase, rho small monomeric GTPase, MAPK related ESTs signifies a signaling network probably regulated GA biosynthesis under MeJA treatment. But the proper functional characterizations of such genes are still pending. Thus, further characterization of those genes involved in the regulation of GA biosynthesis would lead to an in-depth understanding of GA biosynthesis regulation network.

**Materials and Methods**

**Fermentation Conditions and Methyl Jasmonate Elicitation of G. lucidum**

G. lucidum, strain HG, was grown at 28°C in potato dextrose agar (PDA) medium. The fermentation conditions of G. lucidum were maintained as described [12]. For methyl jasmonate induction, MeJA (Sigma, USA) was dissolved in ethanol and were maintained as described [12]. For methyl jasmonate agar (PDA) medium. The fermentation conditions of added to all cultures.

**RNA Extraction Procedure**

For each sample, ~0.5 g of mycelia was collected by filtration from the culture media, dehydrated in liquid nitrogen and stored at −80°C. Total RNA was extracted using an RNA Isolation Kit (Takara, China) and treated with DNase I (Takara, China) according to the manufacturer's instructions.

**cDNA-AFLP Analysis**

The cDNA-AFLP protocol was described previously by Vuylsteke et al. [59]. Double-stranded cDNA was synthesized from 2.5 μg of total RNA using an M-MLV RTase cDNA Synthesis Kit (Takara, China) and an oligo-dT primer (Takara, China).

After pre-amplification, the mixture was diluted 600-fold, and 5 μl was used for selective amplification with each of 64 primer combinations and two selective nucleotides on the MseI primer (Table S4). Touchdown PCR was performed using the following conditions: 2 min of denaturation at 94°C; 13 cycles of 30 s of denaturation at 94°C, 30 s of annealing starting at 65°C and decreasing by 0.7°C per cycle, and 60 s of extension at 72°C; 23 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 56°C, and 60 s of extension at 72°C; and 5 min at 72°C. Selective amplification products were separated on a 6% polyacrylamide gel for 2.5 h at 115 W and 50°C. Images of TDFs were developed by silver staining. TDFs that showed clear differences in intensity were visualized by the Quantity One software Version 4.6 (Bio-Rad, Hercules CA) to identify up-regulated or down-regulated TDFs.

**Sequence Analysis of cDNA-AFLP Fragments**

The bands corresponding to differentially expressed genes were excised from the gels with a surgical blade, and the eluted DNA was reamplified using the selective amplification primers and the following PCR conditions: denaturation for 15 min at 94°C; 35 cycles of 40 s of denaturation at 94°C, 60 s of annealing at 56°C, and 40 s of extension at 72°C; and 5 min at 72°C. The quantity of each reamplified band was assessed on a 2% agarose gel, and the DNA was purified from the gel and then sequenced directly using the same primers that were used for the re-amplification or cloned into a pMD-18T vector (Takara, China) and sequenced. Nucleotide and protein sequences were compared to sequences in the available public databases by BLAST sequence alignment. Homology searching was performed against the NCBI databases. The sequences were manually assigned to functional categories based on the analysis of the scientific literature and also with the aid of the information reported for each sequence by the Gene Ontology Consortium [60].

**Real-time RT-PCR Analysis**

Real-time RT-PCR was performed on pools of RNA derived from two independent biological experiments. All samples were examined in triplicate. The samples were prepared as described above for the cDNA-AFLP. Total RNA was treated with RNase-free DNase I (Takara, China) according to the manufacturer's instructions, and 2.5 μg was then used for reverse transcription with Reverse Transcriptase M-MLV (Takara, China). Then, 5 μl of 1:10 diluted cDNA samples was used as the qRT PCR template with 0.5 μM gene-specific primers and 10 μl SYBR Premix Ex Taq II (Takara, China) in a total volume of 20 μl. All samples were examined in triplicate. Experiments were performed in a Realplex2 Systems (Eppendorf, Germany) with the following thermal cycling profile: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Each real-time assay was tested in a dissociation protocol to ensure that each amplicon was a single product. The relative quantification of gene expression was performed using the housekeeping gene 18S rRNA [61]. Specific primer pairs were designed for the 25 transcriptionally derived fragments (TDFs) chosen for validation using the Primer 3 software (Table S5). The Ct was used to calculate the fold changes (FC) in each gene compared to the expression level detected in the control:

$$\text{DD} = \text{Ct} - \text{Ct}$$

where ΔΔCt = (ΔCt target − ΔCt 18S rRNA) control sample. Gene expression was evaluated by calculating the difference between the Ct of the gene analyzed and the Ct of the control 18S rRNA. Post-qRT-PCR calculations analyzing the relative gene expression levels were performed according to the 2$^{-\Delta\Delta C_{t}}$ method described by Livak and Schmittgen [62].

**Statistical Analysis**

The significance of samples was determined by analysis of variance, and sample means were separated by the Student’s t-test. Statistical significance was expressed as P<0.05 or P<0.01.

**Supporting Information**

**Figure S1** The distribution of 390 TDF on chromosomes in G. lucidum genome.


**Figure S2** Transcript derived fragments (TDFs) homologies to other known protein found in the *G. lucidum* genome. (DOC)

**Figure S3** Expression patterns of 25 genes in the cDNA-AFLP results. (DOC)

**Table S1** Gene Functional Annotations according to Gene Ontology (GO). (DOC)

**Table S2** Pathway description of TDFs by searching the KEGG PATHWAY database. (DOC)

**Table S3** The genes expressed across MeJA-induction and the different developmental stages. (DOC)

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