Recombinant Expression and Bioactivity Comparison of Four Typical Fungal Immunomodulatory Proteins from Three Main *Ganoderma* Species

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**Abstract**

**Background:** More than a dozen of fungal immunomodulatory proteins (FIPs) have been identified to date, most of which are from *Ganoderma* species. However, little is known about the similarities and differences between different *Ganoderma* FIPs' bioactivities. In the current study, two FIP genes termed *FIP-gap1* and *FIP-gap2* from *G. applanatum*, along with *LZ-8* and *FIP-gsi*, another two representative *Ganoderma* FIP genes from *G. lucidum* and *G. sinense* were functionally expressed in *Pichia*. Subsequently, bioactivities of four recombinant *Ganoderma* FIPs were demonstrated and compared.

**Results:** All the four *Ganoderma* FIP genes could be effectively expressed in *P. pastoris* GS115 at expression levels ranging from 197.5 to 264.3 mg L\(^{-1}\) and simply purified by one step chromatography using HisTrap™ FF prepack columns. Amino acid sequence analysis showed that they all possessed the FIP conserved fragments. The homologies of different *Ganoderma* FIPs were from 72.6 to 86.4%. In vitro haemagglutination exhibited that *FIP-gap1*, *FIP-gsi* and *LZ-8* could agglutinate human, sheep and mouse red blood cells but *FIP-gap2* agglutinated none. Besides, the immunomodulation activities of these *Ganoderma* FIPs were as: *rFIP-gap2 > rFIP-gap1 > rLZ-8* and *rFIP-gsi* in terms of proliferation stimulation and cytokine induction on murine splenocytes. Additionally, the cytotoxic activity of different FIPs was: *rFIP-gap1 > rLZ-8 > rFIP-gsi > rFIP-gap2*, examined by their inhibition of three human carcinomas A549, Hela and MCF-7.

**Conclusions:** Taken together, four typical *Ganoderma* FIP genes could be functionally expressed in *P. pastoris*, which might supply as feasible efficient resources for further study and application. Both similarities and differences were indeed observed between *Ganoderma* FIPs in their amino acid sequences and bioactivities. Comprehensively, *rFIP-gaps* from *G. applanatum* proved to be more effective in immunomodulation and cytotoxic assays in vitro than *rLZ-8* (*G. lucidum*) and *rFIP-gsi* (*G. sinense*).

**Keywords:** *Ganoderma*, Fungal immunomodulatory protein, Recombinant expression, Cytotoxicity, Yeast

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Background

Ganoderma belong to Ganodermataceae, Ganodermales, Basidiomycetes and Kingdom Fungi, which have been recorded for use in Orient over two millennia [1]. Ganoderma has been believed to promote human’s health and longevity by treating very heavy diseases, and regarded as mysterious and auspicious herbs [2, 3]. Modern scientific studies have proven that Ganoderma possess immunity-enhancing, anti-tumor, anti-allergy and anti-virus properties etc. [4–7]. The bioactive components responsible for those activities include polysaccharides, glycopeptides, triterpenes, proteins, lectins, nucleotides, amino acids, sterols and alkaloids [8–12].

For example, three sterols and five triterpenes isolated from G. annulare were found to inhibit the growth of the fungi Microsporum canis and Trichophyton mentagrophytes [13]. And polysaccharides from G. lucidum are extensively well-known for their anti-tumor and immunomodulation activities [14–16]. Recent studies exhibited that G. lucidum polysaccharides could serve as regenerative therapeutic agents to treat cognitive decline associated with neurodegenerative diseases by promoting cognitive function and neural progenitor proliferation [17] whereas four spiro-lactone lanostane triterpenoids isolated from G. calidophilum showed moderate cytotoxic activity against K562, BEL7402, and SGC790 cell lines [18]. Generally, Ganoderma polysaccharides and triterpenes are considered as the main effective gradients. Nevertheless, more and more attention has been paid to bioactive proteins from Ganoderma in recent years [19–22]. Of all those bioactive proteins, fungal immunomodulatory proteins (FIPs) are a novelty-identified protein family, which share some amino acid sequence similarity and immunological response action to immunoglobulins [23]. Ever since the first FIP, known as LZ-8 or FIP-glu, was isolated from the mycelia of G. lucidum by Kino et al. in last century [24], more than fifteen FIPs have been identified from different fungi to date [25–31]. Nine of those FIPs are from Ganoderma species including LZ-8 (FIP-glu, G. lucidum), LZ-9 (G. lucidum), FIP-gts (G. tsuga), FIP-gja (G. japonicum), FIP-gsi (G. sinense), FIP-gmi (G. microsporum), FIP-gas (G. astum), FIP-gbo (G. boninense), FIP-gat (G. atrum), FIP-gap1 and FIP-gap2 (both from G. applanatum) [32–35]. The FIPs consist of 110–114 amino acids with molecular weights of 12–13 kDa, which are rich in Asp and Val but poor in His, Cys and Met. Different FIPs share some similarities in their amino acid sequences, for instance, LZ-8 and FIP-gts are 100% identical, whereas FIP-gmi, FIP-gsi, FIP-gat and FIP-gja share 99% similarities [28, 32]. Biological activities of FIPs have been extensively investigated since their discovery, mainly including hemagglutination, anti-anaphylaxis and anti-tumor [24, 25, 28, 30]. Recent studies revealed that FIP-fve exerted anti-inflammatory effects on OVA-induced airway inflammation by reducing airway remodeling and collagen expression [36]. And FIP-gmi could ablate cancer stemness and cisplatin resistance in oral carcinomas stem cells through IL-6/Stat3 signaling inhibition [37].

It is estimated that there are approximately 200 Ganoderma species around the world, among which more than 80 are identified in China [1–3]. Traditional classification of Ganoderma in China was mainly made according to their morphological characteristics [38]. Zhao et al. divided Ganoderma into two main subgenera, Ganoderma and Elfinxia. Subsequently, they found that subgenus Ganoderma contained two subsections, subsect. Phaeonema (Zizhi) and subsect. Ganoderma (Lingzhi) [39, 40]. Interestingly and coincidentally, FIPs have been identified from G. lucidum (LZ-8 or FIP-glu, Genbank No. M58032.1) and G. sinense (FIP-gsi, Genbank No. AY449805.1), which are two most famous representative species from subsect. Phaeonema and subsect. Ganoderma. Similarly, a pair of novel FIP genes have also cloned from G. applanatum, the typical species of subgenus Elfinxia, in our previous study [34, 35], which were termed as FIP-gap1 (or known as FIP-gap, Genbank No. JN167598.1) and FIP-gap2 (Genbank No. KX591653). However, very little is known about the exact differences and similarities between different Ganoderma FIPs until now. In the current study, those typical Ganoderma FIP genes from the three representative species were functionally expressed in Pichia pastoris GS115 and purified. Subsequently, a series of functional assays were performed using different recombinant Ganoderma FIPs in order to analyze and to compare their bioactivities comprehensively. Our results indicated that differences indeed existed between different Ganoderma FIPs despite of some similarities in their protein sequences and amino acid compositions. These findings will provide data on the biological functions of different Ganoderma FIPs which will help to elucidate the potential application and development of FIPs in biomedical or therapeutic studies.

Results

Sequence alignment and phylogenetic tree analysis

FIPs are highly conserved eukaryotic proteins and different FIPs exhibit homology. Hence, homology between Ganoderma FIPs was primarily analyzed by amino acid sequence alignment using DNAMAN software. The NCBI’s protein BLAST showed that Ganoderma FIPs shared high homology, ranging from 72.6 to 100% (Fig. 1). For example, LZ-8 and FIP-gts, FIP-gja and FIP-gmi were 100% identical. FIP-gsi, FIP-gas, FIP-gia and FIP-gmi were of 98% identity. Furthermore, similarities of FIP-gap1 and FIP-gap2 with LZ-8 were 77.9 and 72.6% in their amino acid sequences. And FIP-gsi shared 78.7 and 76.1% homology with FIP-gap1 and FIP-gap2, respectively (Fig. 1;
Additional file 1). Phylogenetic tree of *Ganoderma* FIPs constructed by MEGA (version 7.0) revealed that FIPs from *Ganoderma* species mainly clustered into three lineages (Fig. 2). Firstly, FIP-gat, FIP-gsi, FIP-gja and FIP-gmi formed a big separate lineage. While LZ-8, FIP-gts and LZ-9 clustered into another second lineage. The third lineage included only two FIPs, FIP-gap1 and FIP-gap2, both from *G. applanatum*.

**Gene synthesis and recombinant vector construction**

Four codon-optimized *Ganoderma* FIP genes were artificially synthesized by Sango (Shanghai, China), within...
which a 6 × His tag sequence was also inserted before the stop codon. Codon adaptation indexes (CAI) for Ganoderma FIP genes in P. pastoris before and after codon-optimization are shown in Additional file 2. The sketch map of synthetic FIP genes is as shown in Additional file 3. The eukaryotic expression vectors were constructed via digestion and ligation using synthetic gene fragments and plasmid pPIC9, transformation of E. coli competent cells, and confirmation by colony PCR and sequencing. Finally, recombinant plasmids were obtained and were universally named as pPIC9-His-FIP-Ganoderma (Fig. 3), in which the four Ganoderma FIP genes were integrated under the control of the P AOX promoter.

Expression and purification of Ganoderma FIPs in P. pastoris

After transformation, the correct positive P. pastoris transformants were identified by screening culture on MD (Minimal Dextrose) and colony PCR. The phenotypes of all the yeast transformants were His 'Mut' after further cultivation on MD and MM (Minimal Methanol) media. Growth and induction culture of yeast transformants were conducted in YPD and BMMY, respectively. Purification of recombinant Ganoderma FIPs were performed by one-step chromatography using prepacked HIS Trap™ FF columns. The supernatants from induced culture at 96 h and wild-type yeast, along with the purified Ganoderma FIPs, were subjected to SDS-PAGE and Western blot for detection and analyses. Two protein bands of approximately 14 and 17 kDa could be clearly observed for rFIP-gap1 and rFIP-gap2, whereas single protein bands, 14 and 17 kDa, of rLZ-8 and rFIP-gsi were obviously visualized, respectively (Fig. 4a). Moreover, all those protein bands could be obviously immunologically detected by anti-6 × His antibody (Fig. 4b). Besides, the expression level of rFIP-gap1, rFIP-gap2, rLZ-8 and rFIP-gsi were 247.4, 197.5, 253.6 and 264.3 mg L⁻¹ measured by densitometric scanning and quantitative analysis.

Haemagglutination and mitogenic activities of recombinant Ganoderma FIPs

Hemagglutination activities of recombinant Ganoderma FIPs (all at 5 μg mL⁻¹) towards three different mammalian red blood cells, which was detected using a microscope at magnification of (16 × 20). Results indicated that rFIP-gap1 and rFIP-gsi were able to agglutinate human, sheep and mouse red blood cells, whereas rLZ-8 showed hemagglutination ability to sheep and mouse blood cells, but not the human's. However, the other FIP from G. applanatum, rFIP-gap2, couldn’t agglutinate any of the red blood cells (Table 1; Additional file 4). In addition, PHA (5 μg mL⁻¹) used as a positive control caused haemagglutination towards human, sheep and mouse red blood cells. The MTT method was applied to examine the stimulatory activities of recombinant FIPs, which revealed that all Ganoderma FIPs could significantly stimulate the cell viability of murine splenocytes at concentrations of 5 μg mL⁻¹ by contrast with the negative control (Fig. 5). Interestingly, rFIP-gap1, rFIP-gap2 and rFIP-gsi were even more effective in
splenocyte mitogenesis than the positive control ConA (5 μg mL\(^{-1}\)).

Examination of induced cytokines release from murine splenic lymphocytes

The immunomodulation abilities of Ganoderma FIPs were demonstrated by inducing secretion of IL-2 and IFN-γ from murine splenocytes measured via ELISA. Results indicated that compared with the negative control, all the four FIPs (5 μg mL\(^{-1}\)) could obviously enhance the expression levels of two cytokines. For IL-2, rFIP-gap1, rFIP-gap2, rLZ-8 and rFIP-gsi increased the release up to 623, 417, 525 and 237 pg mL\(^{-1}\), respectively, while the level of IL-2 secretion induced by ConA (5 μg mL\(^{-1}\)), the positive control, reached 769.5 pg mL\(^{-1}\) (Fig. 6a). For IFN-γ, the induced expression levels by rFIP-gap1, rFIP-gap2, rLZ-8 and rFIP-gsi were 1381, 1624, 606 and 1538 pg mL\(^{-1}\), and ConA could increase the level of IFN-γ release to 1483 pg mL\(^{-1}\) (Fig. 6b).

Additionally, rFIP-gap2 was even superior to ConA in IFN-γ induction.

Cytotoxic effect assessment of recombinant Ganoderma FIPs

The cytotoxic activities of different Ganoderma FIPs were demonstrated by their inhibitory effects towards three types of human cancer cells using a MTT method as described above. And different IC\(_{50}\) were calculated from the cell viability data using SPSS 19.0 software. Results indicated that rFIP-gap1 showed significant cytotoxic effect towards A549, Hela and MCF-7 cells, with IC\(_{50}\) values of 29.89, 8.34 and 12.19 μg/mL, respectively (Fig. 7). rLZ-8 and rFIP-gsi exhibited moderate inhibitory activities to the same cancer cell lines. The IC\(_{50}\) values of rLZ-8 were 21.65, 17.53 and 43.72 μg/mL, whereas those of rFIP-gsi were 68.04, 19.44 and 30.05 μg/mL (Fig. 7). rFIP-gap2, the other FIP from G. applanatum showed the poorest cytotoxicity. The IC\(_{50}\) values of rFIP-gap2 towards A549, Hela and MCF-7 cells were 21.65, 17.53 and 43.72 μg/mL, respectively.

| Sources of red blood cells | PBS | PHA | rFIP-gap1 | rFIP-gap2 | rLZ-8 | rFIP-gsi |
|---------------------------|-----|-----|-----------|-----------|-------|---------|
| Human                     | –   | ++  | –         | –         | –     | ++      |
| Sheep                     | –   | ++  | –         | –         | –     | ++      |
| Mouse                     | –   | ++  | ++        | –         | ++    | ++      |

Note: Haemagglutinating examination of various Ganoderma FIPs including rFIP-gap1, rFIP-gap2, rLZ-8 and rFIP-gsi (all final concentration at 5 μg mL\(^{-1}\)) towards human, sheep and mouse red blood cells. PBS and PHA (5 μg mL\(^{-1}\)) served as negative and positive controls. (+) indicates positive reaction and (-) indicates negative reaction. (++) and (+) denotes 75 and 50% of cells underwent haemagglutination, respectively. All results were from biological duplicate tests.
cancer cell lines were 60.92, 41.05 and > 100 μg/mL, respectively (Fig. 7).

**Discussion**

*Ganoderma* have been applied and recorded in China for thousands of years, which are deemed as precious auspicious herbs and thought to prolong life as well as to cure the dying patients [1, 2]. As estimated, there are over 200 *Ganoderma* species worldwide with more than 80 distributed in China, among which *G. lucidum* is the most well-known [3, 39, 40]. Various components from *Ganoderma* have been proven bioactive including polysaccharides, proteins, glycopeptides, triterpenes etc. Previous studies found that polysaccharides and triterpenes were the two main active substances for *Ganoderma* [8, 11, 40]. In terms of that, Zhao et al. once stated that *G. sinense* was the most valuable and bio-effective species [40]. However, discovery of FIPs revealed that proteins alone from *Ganoderma* could exert very similar bioactivities to polysaccharides and triterpenes, such as...
immunomodulation and anti-tumor activities [23]. Since the first FIP, LZ-8, was identified from *G. lucidum*, seventeen FIPs have been reported to date, among which more than a half are from *Ganoderma* [24–30]. And these *Ganoderma* FIPs shared very high homology in their amino acid sequences (Fig. 1). On basis of the protein sequence alignment and phylogenetic tree analysis of *Ganoderma* FIPs (Fig. 2), we could divide *Ganoderma* into three main groups, Lingzhi (*Ganoderma*), Zizhi (*Phaeonema*) and Shushe (*G. applanatum*) in the current paper. That was very similar and accord-ant with the traditional classification of *Ganoderma* as well as other sorting results using molecular markers [41]. Therefore, we can speculate that all *Ganoderma* probably contain FIPs, which can be potentially used as candidate markers for *Ganoderma* classification.

As reported previously, it is time-consuming and costly to extract FIPs from natural mycelia and fruiting bodies with usually low yield [25, 28]. For example, merely 10 mg of LZ-8 was purified from 300 g wet *G. lucidum* mycelia [24]. Hence, researchers have been making efforts in investigating new methods to obtain recombinant FIPs via bio-engineering techniques. For instance, FIP-gts and FIP-fve have been expressed in *E. coli* and insect cells, with yields of ranging from 5 to 20 mg L$^{-1}$ [28]. Besides, LZ-8 has been expressed in *Bacillus subtilis* and *Lactococcus lactis* at expression levels of 17.4 and 1.24 mg L$^{-1}$, respectively [42]. However, the expression levels of these rFIPs from prokaryotic and insect cells are commonly very low. Furthermore, recombinant FIPs from *E. coli* possessed only 50% bio-activity of native ones [42]. The *P. pastoris* expression system is regarded as an ideal host and extensively employed to express functional proteins of medicinal or commercial values [28, 31]. Generally, the expression levels are high as a result of the *P_{AOX}* promoter, which is reported to be the strongest promoter to date, and is regulated by methanol [43]. Besides, recombinant heterogeneous proteins can be produced extracellularly and secreted into the culture media with the help of α-signal peptide, which effectively facilitates the downstream processing and purification. In addition, cultivation and fer-mentation of *P. pastoris* is simple and economical, making it quite applicable for scale-up fermentation and even industrial production [43, 44]. Given all these ad-\[ags, *P. pastoris* GS115 was adopted as a host to express the four typical *Ganoderma* FIP genes in the current study. Results exhibited that they were all effect-ively expressed after codon-optimization, with expres-sion levels of 197.5–264.3 mg L$^{-1}$ (Fig. 4), which is accordant with previous reports (Table 2) [45]. Additionally, two protein bands of rFIP-gap1 and rFIP-gap2 could be clearly observed in SDSPAGE and Western blotting (Fig. 4). The reason for that was probably due to the post-transcriptional modification including glycosylation, which was a common phenomenon in *P. pastoris* expression system [31, 43]. Potential glycosylation sites were found therein the FIP-gaps’ amino acid sequences, including Asn-Leu-Thr in FIP-gap1 and Asn-Ser. Pro-Thr in FIP-gap2.
A series of biological activities have been reported since the discovery of FIPs, including haemagglutination, anti-allergy, immunomodulation, anti-tumor and anti-virus [23–25, 28]. Nevertheless, quite little is known about the similarities and differences between the Ganoderma FIPs. Thus, bioactivities were examined and compared specifically between FIP-gap1, FIP-gap2, LZ-8 and FIP-gsi, the four rFIPs from three representative Ganoderma, in terms of haemagglutination, mitogenesis, cytokine induction and cytotoxic effect. Primarily, rFIP-gap1 and rFIP-gsi showed strong haemagglutination ability, as they could agglutinate human, sheep and mouse red blood cells (Table 1). And LZ-8 was capable to agglutinate sheep and mouse red blood cells but not the human’s, which is identical to natural LZ-8 and rLZ-8s from E. coli or P. pastoris [44]. However, rFIP-gap2, the second FIP from G. applanatum, could agglutinate none of the three blood cells (Table 1). Later, the immunomodulation activities of these Ganoderma FIPs were evaluated by their mitogenesis and cytokine induction towards murine spleen lymphocytes. Results indicated that rFIP-gap2 had the strongest stimulation to enhance cell viability of mouse splenocytes, whereas rFIP-gap1 and rFIP-gsi showed moderate mitogenesis ability, and rLZ-8 was less effective in mitogenic activity (Fig. 5). For cytokine induction test, the ability of Ganoderma FIPs to increase IL-2 release were as follows: rFIP-gap1 > rLZ-8 > rFIP-gap2 > rFIP-gsi (Fig. 6a), while the activity of different FIPs to induce IFN-γ expression were: rFIP-gap2 > rFIP-gsi > rFIP-gap1 > rLZ-8 (Fig. 6b). Finally, the cytotoxic effects of different Ganoderma FIPs were detected by their inhibition towards different human cancer cells via a MTT method. Results revealed that four Ganoderma FIPs showed better cytotoxic activity towards Hela and MCF-7 cancer cells than lung cancer A549. To be more specific, the inhibitory ability of different Ganoderma FIPs were: rFIP-gap1 > rLZ-8 > rFIP-gsi > rFIP-gap2, estimated by their IC50 towards different cancer cell lines (Fig. 7). Additionally, Ganoderma FIPs showed similar but weaker cytotoxic activities by contrast with some other FIPs, for example, FIP-lrh from Lignosus rhinocerotis, exhibited stronger cytotoxicity to A549, Hela and MCF-7 cancer cells, with IC50 values ranging from 5.07 to 8.94 μg/mL [46]. And the IC50 value of another FIP, FIP-sch3 (Stachybotrys chartarum), was 10.80 μg/mL [29]. Besides, the cytotoxicity of rFIP-gaps towards A549 and Hela cells in current study was accordant with those in previous reports with very similar IC50 values [35].

Conclusively, four FIPs including FIP-gap1, FIP-gap2, LZ-8 and FIP-gsi from three representative Ganoderma species were functionally and effectively expressed in P. pastoris in present paper, with expression levels of 197.5 to 264.3 mg L−1. And those recombinant Ganoderma FIPs possessed ideal bioactivities in vitro. The immunomodulation ability of different Ganoderma FIPs was: rFIP-gap2 > rFIP-gap1 > rLZ-8 & rFIP-gsi, whereas the cytotoxicity activity of them was: rFIP-gap1 > rLZ-8 > rFIP-gsi > rFIP-gap2.

**Conclusion**

In the current study, a pair of novel FIP genes from G. applanatum, along with another two FIP genes from G. lucidum and G. sinense, were functionally expressed in

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**Table 2** Recombinant FIPs produced in *Pichia pastoris*

| FIPs | Pichia strains | Vectors | Yields (mg L−1) | Bioactivities | References |
|------|----------------|---------|----------------|--------------|------------|
| LZ-8 | GS115          | pPIC-9  | 191.2          | hemagglutination, mitogenesis, IL-2 induction, anti-tumor | Lin et al., 2009 |
| GS115| pPIC-9 K       |         | 270            | hemagglutination, mitogenesis | Xue et al., 2008 |
| X-33 | pPICZaA        |         | 350            | not mentioned | Bastiaan et al., 2013 |
| X-33 | pPICZaA        |         | 34.4           | hemagglutination | Song et al., 2011 |
| GS115| pPIC-9         |         | 253.6          | hemagglutination, mitogenesis, IL-2 & IFN-γ induction, anti-tumor | Current study |
| FIP-gve| GS115         | pPIC-9  | 158.2          | hemagglutination, mitogenesis, IL-2 & IFN-γ induction | Lin et al., 2013 |
| X-33 | pPICZaA        |         | 189            | hemagglutination | Bastiaan et al., 2013 |
| FIP-vvo| X-33          | pPICZaA | 410            | hemagglutination, mitogenesis, IFN-γ induction | Sun et al., 2014 |
| FIP-nha| X-33          | pPICZaA | 42.7           | hemagglutination | Bastiaan et al., 2013 |
| FIP-cro| GS115         | pPIC-9  | 148.5          | hemagglutination, mitogenesis, IL-2 induction | Lin et al., 2016 |
| LZ-9 | X-33           | pPICZaA | 6.7            | hemagglutination | Bastiaan et al., 2013 |
| FIP-gsi| GS115         | pPIC-9  | 264.3          | hemagglutination, mitogenesis, IL-2 & IFN-γ induction, anti-tumor | Current study |
| FIP-gap1| GS115         | pPIC-9  | 247.4          | hemagglutination, mitogenesis, IL-2 & IFN-γ induction, anti-tumor | Current study |
| FIP-gap2| GS115         | pPIC-9  | 197.5          | hemagglutination, mitogenesis, IL-2 & IFN-γ induction, anti-tumor | Current study |

LZ-8, FIP-vve, FIP-vvo, FIP-nha, FIP-cro, LZ-9, FIP-gsi, FIP-gap1 and FIP-gap2 represent FIPs from *G. lucidum, Flammulina velutipes, Volvariella volvacea, Nectria haematococca, Chroogomphis rutilus, G. lucidum, G. sinense and G. applanatum*.
P. pastoris GS115, at yields ranging from 197.5 to 264.3 mg L\(^{-1}\), which were with much higher yield than recombinant FIPs from E. coli and insect cells. Bioactivity examination of these four typical Ganoderma FIPs indicated that FIP-gap1 and FIP-gap2 showed more immunomodulation activity than LZ-8 and FIP-gsi in terms of mitogenesis and cytokine induction from mouse splenocytes. Besides, cytotoxic effects of these FIPs were: mitogenesis and cytokine induction from mouse splenocytes. Furthermore, our results showed that similarities and differences indeed existed between different Ganoderma FIPs in both their protein sequences and bioactivities. And it seemed that FIPs from G. applanatum were more bioactive than FIPs from G. lucidum and G. sinense. Additionally, our study also confirmed that recombinant FIPs from P. pastoris could be used as robust effective resources for further development and investigation.

Materials and methods


ded synthesis from the synthetic FIP genes and plasmid pPIC9 were digested with EcoRI and NotI at 37 °C for 1.5 h. Then a rapid ligation using target genes and digested pPIC9 was conducted at 22 °C for a half hour. The ligated product was used to transform E. coli TOP10 competent cells, which were later screened on LB media with ampicillin (100 μg mL\(^{-1}\)). The positive E. coli transformants were detected by colony PCR and endonuclease digestion. Finally, the correct recombinant plasmids were obtained and designated as pPIC9-His-FIP-Ganoderma, which were further confirmed by sequencing.

Transformation and induced expression of Ganoderma FIPs

Preparation and transformation of yeast competent cells were performed according to the kit instruction (Catalog number: T2001). Phenotype of yeast transformants was determined by screening on MD and MM media. Positive transformants were verified by colony PCR and cultured in YPD at 30 °C, 200 rpm until OD\(_{600}\) ≥ 2.0. Then the yeast cells were harvested by centrifugation at 6000 rpm for 5 min and then resuspended in BMMY 200 mL (500 mL in one flask) to induce protein expression. Methanol was added every 24 h to maintain a final concentration of 1% (v/v). At 96 h, 1 mL of the expression cultures were transferred for later use. The wild-type yeast was used as a negative control and cultured in a same way. Purification of recombinant Ganoderma FIPs was carried out using HisTrap FF prepack columns according to the manufacturer’s protocol (Catalog number: 17–5319-01). The protein masses of recombinant proteins were determined through SDS–PAGE and quantitative analyses with an image-analyzing system (QUANTITY ONE, Bio-Rad, USA) [28].

SDS-PAGE and Western blot analyses

25 μL supernatants of four recombinant Ganoderma FIPs (at induction time of 96 h) and 10 μL purified protein samples were mixed with equal volumes of 2 × loading buffer for SDS–PAGE test. The wild-type yeast supernatant was as a negative control. All the mixtures were boiled for 5 min before electrophoresis. SDS–PAGE was performed in 12% acrylamide gels and visualized by staining with Coomassie brilliant blue R250. For Western blot, after SDS–PAGE, proteins samples were transferred onto a PVDF membrane. After blocking with 5%
nonfat dry milk in TBST and incubated with anti-His monoclonal antibody raised in mouse (Tiangen, China, 1:25000 diluted) for two hours. The membrane was washed five times with TBST and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG raised in goat (Tiangen, 1:5000 diluted) for 1.5 h at 37 °C [43]. After incubation with the ECL reagent (Pierce, USA), the membrane was subjected to X-ray film for exposure 2–5 s at totally dark conditions. Subsequently, the X-ray film was developed in the developer bath for 15–20 s, and then fixed in the fixer solution.

Haemagglutination and mitogenesis assays of recombinant FIPs
After purification, the agglutination activity of recombinant Ganoderma FIPs was primarily tested. 50 µL of purified rFIP-gap1, rFIP-gap2, rLZ-8 and rFIP-gsi (final concentration of 5 µg mL⁻¹) in PBS were mixed with equal volumes of human, sheep and mouse red blood cells (1.5%, v/v) in a 96-well microplate, respectively. PHA (5 µg mL⁻¹) served as a positive control, whereas PBS was used as a negative control. The plate was incubated in a 5% CO₂ incubator at 37 °C after gently mixed and haemagglutination was examined after 1.5 and 24 h. The mitogenic activity of Ganoderma FIPs was demonstrated by their stimulatory effect towards mouse splenocytes. Homogenous splenocytes of male Balb/c mice (4–6 weeks old, purchased from the Pharmacology Experimental Center of Shenyang Agriculture University) were grown and suspended to 1 × 10⁶ cells mL⁻¹ in RPMI 1640 medium. Subsequently, 100 µL of cell suspension and recombinant Ganoderma FIPs (5 µg mL⁻¹) were seeded into a 96-well plate. Concanavalin A (ConA, 5 µg mL⁻¹) and PBS served as positive and negative controls. The cell viability was evaluated by a MTT method. In brief, after the cells were cultured in 5% CO₂ for 48 h at 37 °C, 20 µL MTT (5 µg mL⁻¹) was added, and the cell mixtures were incubated for another 4 h. The cell supernatants were carefully removed and 100 µL dimethyl sulfoxide (DMSO) was added into the plate. The plate was then read on a microplate reader (Model 680, Bio-Rad) at 570 nm. The experimental procedures have been approved by the Ethics Committee for Laboratory Animal Care (Animal Ethics Procedures and Guidelines of the People’s Republic of China) for the use of Shenyang Agricultural University, China. (Permit No. 264 SYXK<Liao> 2011–0001).

Cytokines induction detection
To test the immunomodulatory bioactivities of recombinant Ganoderma FIPs, the expression levels of induced IL-2 and IFN-γ released from the murine splenocytes were detected by ELISA method. The murine splenocytes were prepared as above and adjusted to 1 × 10⁷ cells mL⁻¹ in RPMI 1640 medium. Then 100 µL cells plus equal volumes of rFIP-gap1, rFIP-gap2, rLZ-8 and rFIP-gsi (final concentration of 5 µg mL⁻¹) were mixed and seeded into a 96-well plate. Meanwhile, PBS and ConA (5 µg mL⁻¹) were used as controls. After the plate was cultured in 5% CO₂ at 37 °C for 48 h, the supernatants of the cells were collected. Expression levels of mouse IL-2 and IFN-γ in the supernatants were measured using ELISA kits (Multi Sciences, Hangzhou, China) in accordance with the manufacturer’s instructions, and serial concentrations (7.81, 15.63, 31.2, 62.5, 125 and 250 pg mL⁻¹) and (62.50, 125, 250, 500, 1000 and 200 pg mL⁻¹) of mouse IL-2 and IFN-γ were prepared as standards, respectively [28, 43].

Cytotoxicity examination of recombinant Ganoderma FIPs
To determine cytotoxic activities, three cancer cell lines, human lung carcinoma A549, cervical carcinoma Hela and human breast carcinoma MCF-7 were used for test, which were provided by the Cell Research Center, College of Veterinary Science and Animal Husbandry (SYAU). Those cancer cells were cultured in DMEM (Tiangen) supplemented with 10% FBS, 100 units/mL penicillin and 100 µg mL⁻¹ streptomycin at 37 °C in a 5% CO₂ incubator. Subsequently, the cells were trypsinized with 0.25% trypsin containing 0.04% EDTA and adjusted to 5 × 10⁵ cells mL⁻¹. 100 µL cancer cells were seeded in triplicate wells with diluted rFIP-gap1, rFIP-gap2, rLZ-8 and rFIP-gsi (final concentration of 2, 4, 8, 16 and 32 µg mL⁻¹), which were continually cultured for another 24 h. Meanwhile, PBS was added into these cell cultures in a same way as a negative control. Inhibition of cancer cell growth was presented using the half-maximal inhibitory concentration (IC₅₀), which was defined as the concentration causing 50% inhibition of cell proliferation [28].

Data analysis
Data were presented as the mean ± SD of three separate experiments performed in duplicate. Statistical analysis was performed through means of one-way ANOVA and the LSD-test with SPSS 19.0 software. Differences were considered to be statistically significant when the P < 0.05.

Additional files

Additional file 1: Homology between different Ganoderma FIPs based on their peptide sequence alignment using a DNAman software (Version 8.0). FIP-gap1 and FIP-gap2, FIP-gat, FIP-gbo, FIP-gja, FIP-gmi, FIP-gsi, FIP-gts, LZ-8 and LZ-9 represented FIPs from Ganoderma species. G. japonicum, G. microsporum, G. sinense, G. tsugae and G. lucidum, respectively. (DOCX 411 kb)

Additional file 2: Codon adaptation indexes (CAI) for Ganoderma FIP genes in P. pastoris. FIP-gap1, FIP-gap2, LZ-8, and FIP-gsi represent FIP genes from G. applanatum, G. lucidum and G. sinense. (DOCX 13 kb)
Competing interests
Consent for publication
Not applicable.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article or is available from the corresponding author on reasonable request.

Declaration
Some of the assays and methodologies in the Methods section have been published previously [35].

Authors’ contributions
Q-ZW, Z-SY, G-SX, G-R, D-ZW and Z-X performed the experiments. Q-ZW drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
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References
1. Mao XL. The Macrofungi in China. Zhengzhou, Henan: Science and Technology Press; 2000. p. 483–96.

2. Xu JT. Chinese medical mycology. Beijing: Peking Union Medical College Press; 1997. p. 40–2.

3. Chang S, Buswell JA. Ganoderma lucidum (Curt.: Fr.) P. Karst. (Aphyllophoromycetidae)—a mushrooming medicinal mushroom. Int J Med Mushrooms. 1999;1:139–46.

4. Wang YY, Khoa KH, Chen ST, Lin CC, Wong CH, Lin CH. Studies on the immuno-modulating and antitumor activities of Ganoderma lucidum (Reishi) polysaccharides: functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. Bioorg Med Chem. 2002;10:1057–62.

5. Lin ZB. Cellular and molecular mechanisms of immunomodulation by Ganoderma lucidum. J Pharmacol Sci. 2005;99:144–53.

6. Zhang W, Tao J, Yang X, Yang Z, Zhang L, Liu H, Wu K, Wu J. Antiviral effects of two Ganoderma lucidum triterpenoids against enterovirus 71 infection. Biochem Biophys Res Commun. 2014;449:307–12.

7. Ergun B. Evaluation of antimicrobial, cytotoxic and genotoxic activities of Ganoderma lucidum (Reishi mushroom). Pak J Pharm Sci. 2017;30(Supplement):1991–5.

8. Ferreira IC, Heleno SA, Reis FS, Stojkovic D, Queiroz MJ, Vasconcelos MH, Sokovic M. Chemical features of Ganoderma polysaccharides with antioxidant, antitumor and antimicrobial activities. Phytochemistry. 2015;14:38–55.

9. Chang UM, Li CH, Lin LI, Huang CP, Kan LS, Lin SB. Ganoderol F, a Ganoderma triterpene, induces senescence in hepatoma HepG2 cells. Life Sci. 2006;79:1129–39.

10. Ngai PH, Ng TB. A mushroom (Ganoderma capense) lectin with spectacular thermostability, potent mitogenic activity on splenocytes, and antiproliferative activity toward tumor cells. Biochem Biophys Res Commun. 2004;314:988–93.

11. Jiang Y, Chang Y, Liu Y, Zhang M, Luo H, Hao C, Zeng P, Sun Y, Wang H, Zhang L. Overview of Ganoderma sinense polysaccharide-an adjunctive drug used during concurrent chemo/radiation therapy for cancer treatment in China. Biomed Pharmacother. 2017;96:685–70.

12. Gill BS, Naqvi MMA, Raza S, Khan SI. Antifungal activity of sterols and triterpenes isolated from Ganoderma annulare. Fitoterapia. 2003;74:375–7.

13. Yang G, Yang L, Zhang Y, Qian X, Shen Y, Ganoderma lucidum polysaccharide exerts anti-tumor activity via MAPK pathways in HL-60 acute leukemia cells. J Signal Transduct. Res. 2016;6:6–13.

14. Meng LZ, Xie J, Lv GP, Hu DJ, Zhao J, Duan JA, Li SP. A comparative study immunomodulatory activity of polysaccharides from two official species of Ganoderma (Lingzhi). Nutr Cancer. 2011;64:121–34.

15. Xu X, Chen D, Yang L, Zhu N, Li J, Zhao J, Hu Z, Wang FL, Zhang LW. Comparative studies on the immunoregulatory effects of three polysaccharides using high content imaging system. Int J Biol Macromol. 2016;86:28–42.

16. Huang S, Mao J, Ding K, Zhou Y, Zeng X, Yang W, Wang P, Zhao C, Yao J, Xia P, Pei G. Polysaccharides from Ganoderma lucidum promotes cognitive function and neural progenitor proliferation in mouse model of Alzheimer’s disease. Stem Cell Reports. 2017;8:884–94.

17. Huang SZ, Ma QY, Kong FD, Guo ZK, Cai CH, Hu LL, Zhou LM, Wang Q, Dai HF, Mei WL, Zhao YX. Lanostane-type triterpenoids from the fruiting body of Ganoderma caldiphilum. Phytochemistry. 2017;143:104–10.

18. Wang H, Ng TB, Ganodermin, an antifungal protein from fruiting bodies of the medicinal mushroom Ganoderma lucidum. Peptides. 2006;27:27–30.

19. Du M, Wang C, Hu XS, Zhao GH. Biological properties of different protein extracts from selenium-enriched Ganoderma lucidum. Int J Food Sci Nutr. 2008;59:134–47.

20. Xu XF, Yan HD, Chen J, Zhang XW. Bioactive proteins from mushrooms. Biotechnol Adv. 2011;29:667–74.

21. Wong KL, Wong RN, Zhang L, Liu WK, Ng TB, Shaw PC, Kwok PC, Lai YM, Zhang ZJ, Zhang Y, Tong Y, Cheung HP, Lu J, Sze SC. Bioactive proteins and peptides isolated from Chinese medicines with pharmaceutical potential. Chin Med. 2014;9:119.

22. Li QZ, Wang XF, Zhou XW. Recent status and prospects of the fungal immunomodulatory protein family. Crit Rev Biotechnol. 2011;31:365–75.
25. Ko JL, Hsu CJ, Lin RH, Jai CL, Lin JY. A new fungal immunomodulatory protein, FIP-aye isolated from the edible mushroom, Flammulina velutipes and its complete amino acid sequence. Eur J Biochem. 1995;228:244–9.

26. Ko JL, Hsu CI, Lin RH, Jai CL, Lin JY. Dimerization of the N-terminal amphipathic-helix domain of the fungal immunomodulatory protein from Ganoderma tsugae (Fip-gts) defined by a yeast two-hybrid system and site-directed mutagenesis. J Biol Chem. 1997;272:20044–8.

27. Li SY, Shi Li, Ding Y, Nie Y, Tang XM. Identification and functional characterization of a novel fungal immunomodulatory protein from Postia placenta. Food Chem Toxicol. 2015;78:64–70.

28. Lin JW, Guan SY, Duan ZW, Shen YH, Fan WL, Chen L, Zhang L, Li TL. Gene cloning of a novel fungal immunomodulatory protein from Chroogomphus rhinocerots and its expression in Pichia pastoris. J Chem Technol Biotechnol. 2016;91:2761–8.

29. Li S, Zhao L, Xu W, Jiang Z, Wang F, Xin F. Identification and characterization of a novel fungal immunomodulatory protein, FIP-sch3 from Stachybotrys chartarum. PLoS One. 2015;11:e0168436.

30. Li S, Jiang Z, Sun L, Liu X, Huang Y, Wang F, Xin F. Characterization of a new fungal immunomodulatory protein, FIP-dsq2 from Dichomitus squalens. J Biotechnol. 2017;246:45–51.

31. Basitaa-Net S, Chanput W, Hertz A, Zweitink RD, Mes JJ, Wiggers HJ. Biochemical and functional characterization of recombinant fungal immunomodulatory proteins (FIPs). Int Immunopharmacol. 2013;13:167–75.

32. Li QZ, Wang XF, Chen YY, Fan WL, Chen LJ, Zhang L, Li TL. Cytokines expression induced by Ganoderma applanatum immunomodulatory proteins from Ganoderma applanatum (FIP-gts) in mouse spleen cells. Appl Biochem Biotechnol. 2010;162:1403–13.

33. Xu H, Kong YY, Chen X, Guo MY, Bai XH, Lyu Y, Li W, Zhou XW. Recombinant FIP-gat, a fungal immunomodulatory protein from Ganoderma atum, induces growth inhibition and cell death in breast cancer cells. J Agric Food Chem. 2016;64:2690–8.

34. Lin JW, Duan ZW, Guan SY, Han X, Fan WL, Li HG, Zhang L, Chen SS, Li TL. Cloning G. Bioinformatic analysis and eukaryotic expression vector construction of FIP-gap Fene from Ganoderma applanatum. Journal of Shenyang Agricultural University. 2016;47:1–7.

35. Zhou SY, Guan SX, Duan ZW, Han X, Zhang X, Fan WL, Li HG, Chen LJ, Ma H, Liu HM, Ruan YJ, Lin JW. Molecular cloning, codon-optimized gene expression and bioactivity assessment of two novel fungal immunomodulatory proteins from Ganoderma applanatum in mouse spleen cells. Appl Biochem Biotechnol. 2018;102(13):5483–94.

36. Lee YT, Wu CT, Sun HL, Ko JL, Lue KH. Fungal immunomodulatory protein could modulate airway remodeling by affect IL17 cytokine. J Microbiol Immunol Infect. 2017;50(11):S1684-1182(17):30121-4.

37. Wang TY, Yu CC, Hsieh PL, Liao YW, Yu CH, Chou MY. GM1 ablates cancer stemness and cisplatin resistance in oral carcinomas stem cells through IL6/Stat3 signalling inhibition. Oncotarget. 2017;8:70422–30.

38. Zhang QX, Zhao JD. Chinese Flora, Beijing, volume 18. Ganodermataceae: World J Microbiol Biotechnol. 2009;25:383–90.

39. Zhao JD, Xu LW, Zhang XQ. Studies on the taxonomy of Ganodermataceae in China. Mycosystema. 1984;25:439–45.

40. Yeh CM, Yeh CK, Hsu XY, Luo QM, Lin MY. Extracellular expression of a functional recombinant Ganoderma lucidum immunomodulatory protein by Bacillus subtilis and Lactococcus lactis. Appl Environ Microbiol. 2008;74:1309–49.

41. Lin JW, Hua L, Xu GX, Sun F, Gao F, Zhang R, Liu LX. Molecular cloning and recombinant expression of a gene encoding a fungal immunomodulatory protein from Ganoderma lucidum in Pichia pastoris. World J Microbiol Biotechnol. 2009;25:383–90.