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Advances in Biochemistry and Biotechnology

Wang Z, et al. Adv Biochem Biotechnol 2017: 115.

Research Article

Expression of Mcpgip1 and Mcpgip2 in the Momordica charantia Stem Base and Their Eukaryotic Expression

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Citation: Wang L, Qin S, SuoY, Li Y, Ji C, et al. (2017) Expression of Mcpgip1 and Mcpgip2 in the *Momordica charantia* Stem Base and Their Eukaryotic Expression. Adv Biochem Biotechnol 2017: 115

Received Date: 19 January 2017; **Accepted Date:** 31 January 2017; **Published Date:** 7 February 2017

Abstract

Background: Polygalacturonase-Inhibiting Proteins (PGIPs) are defence proteins that can specifically recognize and limit fungal and insect Polygalacturonases (PGs). Much research has shown that pgip over-expression can improve resistance. In this study, we found two pgips, Mcpgip1 and Mcpgip2, in *Momordica charantia* for the first time, along with the expression of two pgips in *Pichia. pastoris*.

Results: RT-PCR and Thermal Asymmetric Interlaced PCR (TAIL-PCR) were used to harvest two pgip genes, Mcpgip1 and Mcpgip2, in *M. charantia*. The fragment cDNA lengths of Mcpgip1 and Mcpgip2 were 987 and 1005 bp, respectively. The invading roots of plants were infected using *Fusarium oxysporum f. sp. momordicae* (Fom), and expression in the stem base was detected using real-time quantitative PCR. McPGIP1 and McPGIP2 expression levels increased. The two pgips were cloned into pPICZaA and expressed in *P. pastoris*. It can produce proteins when alcohol is added to the medium, and SDS-PAGE and Western blot were used to detect the target proteins from the two pgip genes expressed in *P. pastoris*. Dinitrosalicylic Acid (DNS) reagent was used to detect enzyme activity in the inhibition assays. The results confirmed the crude activities of McPGIP1 and McPGIP2 against PGs from *Aspergillus niger*.

Conclusions: *M. charantia* has two pgip genes, the expression levels of which increase under Fom induction. The two genes can be expressed in *P. pastoris*, and the crude proteins can inhibit PGs from *A. niger*.

Keywords: Eukaryotic expression; *Momordica charantia*; Polygalacturonase Inhibiting Protein; Real-time PCR

Abbreviations

ePGs	:	Endopoly-Galacturonases
PGIP	:	Polygalacturonase Inhibiting Proteins
TAIL-PCR	:	Thermal Asymmetric Interlaced PCR
RACE	:	Rapid Amplification Of Cdna Ends
qPCR	:	Real-Time Quantitative PCR
LRR	:	Leucine-Rich Repeat

Introduction

Plants possess a polysaccharide-rich cell wall that acts as a barrier to pathogenic fungi [1]. Many hydrolytic enzymes are secreted to hydrolyse the homogalacturonan component of the plant cell wall when fungal pathogens invade the plant. Endo Poly Galacturonases (ePGs) are the first enzymes to be detected in the plant cell wall during plant fungal infection, suggesting that ePGs play an important role in the virulence of pathogenic fungi [2,3]. The elimination of an endoPG in the saprophytic fungus *Aspergillus flavus* resulted in a mutant with reduced virulence towards cotton bolls [4]. Ten Have A et al. verified that the ePG gene Bcpg1 is required for full virulence of *Botrytis cinerea* [5].

It is well known that in plant-pathogenic interaction systems, plants can secrete plant Polygalacturonase-Inhibiting Pro-

teins (PGIPs), which can specifically recognize and inhibit fungal and insect PGs [6,7]. Research has shown that the PGIPs belong to the Leucine-Rich Repeat (LRR) proteins and have the same 24-residue motif: xxLxLxxNxLt/GxIPxxLxxLxxL. The LRR forms the hydrophobic core, while the side chains of the amino acids flanking the leucine residues are exposed to the solvent and interact with the PGs [8,9].

There are two ways that PGIPs can improve the defence response of plants. First, the PGs–PGIP interaction favours the accumulation of the elicitor-active oligogalacturonides and causes the activation of defence responses. Second, PGIPs can inhibit PG activity [10]. It appears that the PGIPs display differential inhibition towards PGs, not only from various fungi but also towards different isoforms of PGs that originate from a specific pathogen [1]. VvPGIP1 exhibits differential inhibition of the PGs of *A. niger* and *B. cinerea* and differential inhibition of PGs from *B. cinerea* [11]. Over-expressed MdPGIP1 in tobacco showed differential inhibition of PGs from *Botryosphaeria obtusaa* and *Diaporthe ambigua*, but there was no action towards PGs from *A. niger* [12]. PGIPs in pearl millet are involved in resistance against downy mildew [13]. *Brassica napus* PGIPs inhibit *Sclerotinia sclerotiorrum* ePG enzymatic activity and necrosis and delay symptoms in transgenic plants [8]. Over expressed GmPGIP3 in wheat enhances resistance to both take-all and common root-rot disease [14].

De Lorenzo et al. reported that the genes encoding PGIPs are organized by family [1]. Different plants have different numbers of PGIPs; only one is present in soybeans, and five are present in beans [7]. Two PGIP genes are present in *Arabidopsis thaliana* [15], and B. napus possesses a greatly expanded set of 16 or more PGIP genes [16].

Under native conditions, PGIP expression levels are very low, and when plants are induced by pathogens or stress, their expression levels are up-regulated, though they differ in different tissues and development stages [17]. It has been suggested that in ginseng, the expression of mRNA transcripts of PGIP genes in roots was higher than in any other organs [18]. This is profile similar to the expression of Tapgip1 and Tapgip2 in wheat and that of Ospgip1 in rice, which is also highest in roots [19].

No reports have characterized the pgip genes of *M. charantia*. To harvest more pgip genes and to more efficiently filter PGIPs to inhibit PGs. two pgip genes from *M. charantia* were identified using Thermal Asymmetric Interlaced PCR (TAIL PCR) and random amplification of cDNA ends PCR (RACE-PCR). Real-time fluorescent quantitative technology was used to detect the expression levels of PGIPs in *M. charantia* infected using two specialized types of *Fusarium oxysporum*. PGIPs were expressed in *Pichia pastoris*, and Western blot was used for confirmation.

Materials and Methods

Plant Materials

The *M. charantia* (No. 3 of fenglv) seeds used in this study were obtained from the Vegetable Research Institute of the Agricultural Sciences Academy, Guangdong Province.

Fungal Materials

Fusarium oxysporum f. sp. *momordicae* (Fom) and *Fusarium oxysporum* f. sp. *cubense race* 4 (Foc4) were all saved by our laboratory.

TAIL PCR Harvest of the Full-Length cDNA of Mcpgip1

Total DNA was extracted from plant leaves using a Plant DNA kit (OMEGA, USA). We designed a pair of degenerate primers on the basis of the pgip sequences of Rosaceae plants, as recorded in the NCBI database. The degenerate primers were as follows:

F: 5'-AYCCCTACRTYYTKDCCTCATGGAA-3';

R: 5'-ACCRCACARRCABYKGTTATGRAA-3'.

Total DNA was used as template, and degenerate primer and PCR mixture were added. The first step was 94°C for 5 min for one cycle; the second step was 94°C for 30 s, 52°C for 30 s and 72°C for 1 min for 30 cycles; and the last step was 72°C for 10 min. We then harvested and sequenced the product (Invitrogen, USA). The flanking sequence primers of TAIL-PCR were designed using the sequence results, and the primer sequences are shown in Table 1.

Primer	Direction 5'-3'
SP1-R	CTGACAGTTGGTTGTGGGAGAGGTAA
SP1-F	TTACCTCTCCCACAACCAACTGTCAG
SP2-F	ACGATGGACTCCAGTCCGGCCGTTGAAGGAGAG-GTCGAGGTAAGTG
SP2-R	ACGATGGACTCCAGTCCGGCCGTCTAAGGTTGT-GTTTCCTCGGAGTG
SP3-F	TTGTGGAGCATCAGGGTTTCGAGGA
SP3-R	GGTTGCAGAGGTTTGATGTCTATGAG
LAD1-1	ACGATGGACTCCAGAGCGGCCGC(G/C/A)N(G/C/A) NNNGGAA
LAD1-2	ACGATGGACTCCAGAGCGGCCGC(G/C/T)N(G/C/T) NNNGGTT
LAD1-3	ACGATGGACTCCAGAGCGGCCGC(G/C/A)(G/C/A) N(G/C/A)NNNCCAA
LAD1-4	ACGATGGACTCCAGAGCGGCCGC(G/C/T)(G/C/T) N(G/C/T)NNNCGGT
AC1	ACGATGGACTCCAGAG

Table 1: The TAIL-PCR primers of the McPGIP1 flanking sequences.

Full-length cDNA harvesting was performed according to Liu, 2007 [20]. During TAIL-PCR, we harvested a full-length cDNA (Mcpgip1) and a partial gene sequence. RACE-PCR was used to harvest the full-length sequence.

Construction of cDNA Library

Five-leaved plants were induced using Fom, and total RNA was extracted using a Plant RNA kit (Omega, USA). RNA was handled as required by the manufacturer's instruction manual (TaKaRa, Japan). A commercial cDNA synthesis kit was used to construct a library according to the manufacturer's instruction manual (TaKaRa, Japan). To obtain the full-length coding sequence of the Mcpgip2 gene, rapid amplification of cDNA ends (RACE) PCR was performed. Specific primers were designed according to the partial sequences as follows:

Primers from 5'-Full RACE

Mcpgip2-5'-outer: 5'-GAAGGAGAGATCGAGAAAGGTG-3';

Mcpgip2-5'-inter: 5'-AGTTGGTCGTCGGCGAAGATGGT-3';

Primers from 3'-Full RACE

Mcpgip2-3'-outer: 5'-GAAAACACTGCGCCACGTAACA-3';

Mcpgip2-3'-inter: 5'-TGGCTACTCCAAACCTCAAGCT-3'.

The RACE-PCR conditions were: 94°C for 5 min for one cycle; 94°C for 30 s, 52°C for 30 s and 72°C for 1 min for 30 cycles; and 72°C for 10 min. The PCR product was analysed using a 1% agarose gel, and DNA bands were recovered using a DNA purification kit (Axygen, USA), sequenced and harvested as full-length sequences.

Stress-Induced Expression

M. charantia plants were cultivated in pots with nutrient soil at 25°C. Plants were used for the expression analysis at the five-leaf stage. These plants were treated with a spore suspension of Fom. Fom was grown on liquid medium containing 10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4.7H_2O$, 0.02 g $FeCl_3$ and 50 g sugar according to Marangon et al. with improvements [21]. The concentration was 106/ml. Each plant was infected with 20 ml of spore suspension through the plant root. The bottoms of plant stems were harvested at 0, 12, 24, 36 and 48 h post-infection, frozen in liquid nitrogen and stored at-80°C.

RNA Isolation and Real-Time Quantitative PCR

RNA was extracted from plants using a Plant RNA kit (OMEGA, USA) according to the instructions given by the manufacturer. RNA quality and concentration was measured using a NanoPhotometer™ Pearl61010-1 (Implen GmbH, Germany). To obtain the first strand of cDNA, 500 ng of total RNA was reverse-transcribed using a commercial cDNA synthesis kit (TaKaRa, Japan) and diluted to 20% using nuclease-free water. Real-time quantitative PCR was performed using the Thermal Cycler Dice Real-time System Software (TaKaRa, Japan) using 2 μL of cDNA in a 20-μL reaction volume using SYBR○RPremix ExTaq™II (Tli RNaseH Plus, TaKaRa, Japan) and the following primers:

qMcpgip1-F: 5'-CCTCCTCTTCATCTCCTCCTCCAT-3'

qMcpgip1-R: 5'-ACGGTGAGAAGTAGGATGACATTGG-3'

qMcpgip2-F: 5'-CCTTTACCTCTCCCACAACCAACT-3'

qMcpgip2-R: 5'-AAACACAACCGCCGTCAAACTG-3'

The housekeeping gene encoding the actin protein was used as a control in the experiment and was amplified with the following primers: forward 5'-GTATGTTGCCATCCAGGCCG-3'; reverse 5'-ATGTCACGGACAATTTCCCGTTC-3'. The PCR conditions consisted of three steps; the first step was 95°C for 30 s; the second step contained 40 cycles, each cycle at 95°C for 5 s and 60°C for 30 s; and the third step was 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Fluorescence was detected using a real-time PCR thermocycler. All real-time PCRs were performed in triplicate. The results were analysed using Thermal Cycler Dice Real-Time System Software (TaKaRa, Japan). Three samples were analysed for each treatment, and values are given in means ± SD.

Expression of *M. Charantia Pgip* Genes in *P. Pastoris*

ORFs encoding Mcpgip1 and Mcpgip2 genes were harvested as described and cloned into pPICZaA (Invitrogen) using the EcoRI and XbaI restriction sites; pPICZaA was an expression vector used to produce proteins in *P. pastoris*. The plasmid includes the Alcohol Oxidase (AOX) promoter, a signal sequence (α-factor) and a hexahistidine tail sequence. In medium containing alcohol, *P. pastoris* can produce proteins containing a hexahistidine tail, and proteins can be secreted by *P. pastoris* in the supernatant. This hexahistidine tail can be used to detect and purify the proteins.

To harvest proteins, host cell preparation and transformation were performed according to the manufacturer's protocol. Cells were grown in 50 ml of buffered complex glycerol medium at 28°C and 200 rpm until an OD600 of 2-6. Cells were transformed into 100 ml of buffer complex 1% alcohol medium by centrifugation and re-suspension. Samples were collected every 12 h until 72 h; cells were removed by centrifugation at 14,000 g for 10 mins, and the supernatant was stored at-80°C.

Western Blot Analysis

For protein detection, supernatant proteins were separated using 12% denaturing SDS-PAGE gels and were transferred to a Poly Vinylidene Fluoride (PVDF) membrane, which was blocked for 1 h in 3% skim milk dissolved in TBST buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1% Tween-20, pH 7.5). The PVDF membrane

was incubated in antihexahistidine antibody (CST, USA) for 1.5 h, followed by goat anti-rabbit horseradish peroxidase secondary antibody (BioRad Laboratories) for 1 h. The antihexahistidine and secondary antibodies were, respectively, used at 1/2000 and 1/3000 dilutions in TBST buffer. After washing the PVDF membrane fully in 1×TBST buffer, horseradish peroxidase signal was detected using an Enhanced HRP-DAB Chromogenic Substrate Kit (TIANGEN, China).

PGIP Inhibition Activity Assay

Dinitrosalicylic Acid (DNS) reagent [22] was used to detect enzyme activity in the inhibition assays. PG activity was expressed as Reducing Units (RUs). One RU was defined as the amount of enzyme required to release reducing groups at 1 μmol min-1 using D-galacturonic acid as a standard. One unit of PGIP was defined as the amount of inhibitor required to reduce one unit of PG activity by 50% [18].

First, the assay detected the PGs. The 1-ml reaction mixture contained 400 μL of 1 mg/ml polygalacturonic acid (PGA, Sigma, USA) and 100 μL of *A. niger* PGs (53 mg/ml, Sigma, USA) (diluted at 1/2000 in 50 mM sodium acetate buffer, pH 5.0, 500 μL of 50 mM pH 5.0 sodium acetate) and incubated at 30°C for 10 min. Finally, 1 ml of DNS was added to complete the reaction. The PGIP assay steps were as follows: first step, 100 μL of *A. niger* PGs and 100 μL of PGIP were incubated at 30°C for 1 h. Next, 400 μL of 1 mg/ml polygalacturonic acid and 400 μL of sodium acetate were added at 30°C for 10 min. For the control, 100 μL of the supernatant proteins of pPICZaA in *P. pastoris* was used. The sample volume was increased to 5 ml using ddH2O, and the absorbance was measured at 575 nm using a spectrophotometer and water as a blank.

Results

Full-length cDNA

By extracting the total DNA of *M. charantia* using TAIL-PCR, we harvested a full-length pgip gene, both Mcpgip1 and a partial sequence. Fom was used to induce the plant and to extract the total RNA. RT-PCR and RACE-PCR were used to amplify the full length of the partial sequence. We acquired another pgip gene to give two pgip genes: Mcpgip1 and Mcpgip2. The fragment cDNA lengths of Mcpgip1 and Mcpgip2 were 987 and 1005 bp, respectively. The two fragments had Open Reading Frames (ORFs) of 328 and 334 amino acids (aa). The Mcpgip1 amino acid sequence had two repeats of a 24-amino-acid LRR domain, and Mcpgip2 also had a repeat domain. The organization of the domain was LxxLxLxxNxLt/GxIPxxLxxLxxL; plant PGIPs generally contain this construct [9] (Di Matteo et al., 2006). Two genes had submitted to NCBI, and received accession numbers for two nucleotide sequences, the number for Mcpgip1 and Mcpgip2 is KY401178 and KY401179, respectively.

PGIP Expression in Plant Organs

The invading roots of plants use Foc4 and Fom, and expression was detected in the stem base using real-time quantitative PCR. We concluded that (Figures 1 and 2) 1: neither McPGIP1 or McPGIP2, the relative expression all up-regulated treated by Fom; 2: after treatment with Fom, the McPGIP1 and McPGIP2 maximum relative expression levels were 2.5 and 2.8; 3: when plants were treated with Fom, the relative expression of McPGIP1 and McPGIP2 reached maximum levels at 36 h; and 4: when plants were treated with Fom, the maximum relative expression was observed at 12 h.

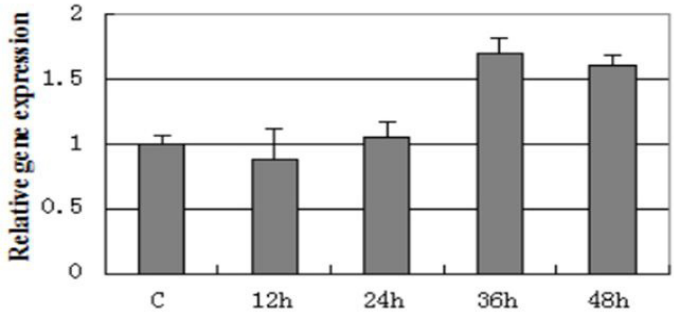

Figure 1: McPGIP1 expression in *Momordica charantia* using Fom; C, 12 h, 24 h, 36 h and 48 h represent *Momordica charantia* infected by Fom and McPGIP1 expression at 0, 12, 24, 36 and 48 h. *Momordica charantia* were infected by Fom, and McPGIP1 reached its highest expression after 48 h.

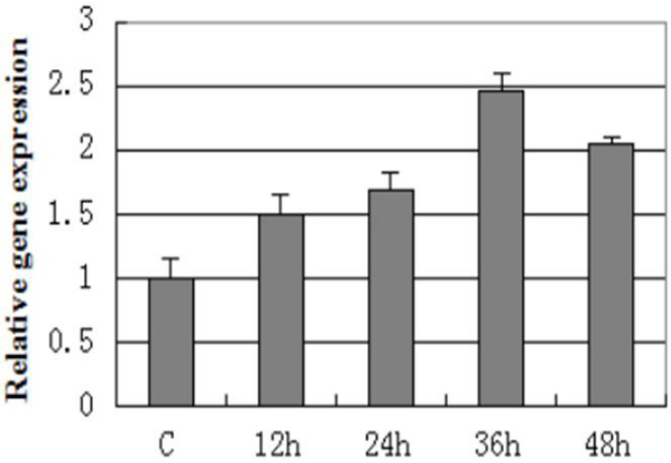

Figure 2: McPGIP2 expression in *Momordica charantia* using Fom; C, 12 h, 24 h, 36 h and 48 h represent *Momordica charantia* infection by Fom and McPGIP2 expression at 0, 12, 24, 36 and 48 h. *Momordica charantia* were infected by Fom, and McPGIP2 reached its highest expression after 36 h.

Expression of *M. charantia* PGIPs in *P. Pastoris*

McPGIP1and McPGIP2 genes were cloned into pPICZa and transformed into *P. pastoris*. The target gene was integrated into the chromosome of *P. pastoris*. Methyl alcohol was used to induce PGIPs, and the liquid supernatant of the yeast was collected. The expressed PGIPs were detected using 12% denaturing SDS-PAGE gels. Gels were stained with Coomassie Blue, and the target proteins were observed between 35 and 55 kD (Figure 3 A and B). Proteins at 0 h were taken as controls. Target protein expression was clearly visible at 12 h.

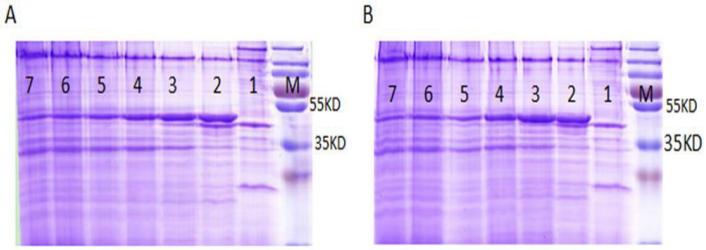

Figure 3: *M. charantia* PGIP expression in *P. pastoris*. Supernatant was harvested every 12 h, and proteins were detected using 12% denaturing SDS-PAGE gels. Part A is the supernatant of McPGIP1; 1-7 represent 0, 12, 24, 36, 48, 60 and 72 h, respectively; Part B is the supernatant of McPGIP2, 1-7 represent 0, 12, 24, 36, 48, 60 and 72 h, respectively. The molecular weights of McPGIP1 and McPGIP2 were both approximately 40 kD, and McPGIP1 and McPGIP2 expression was demonstrated by bands of approximately 40 kD in the gel.

Western blots showed that target proteins were harvested from the supernatant. No protein bands were observed in the supernatant of pPICZaA. The expression of target proteins was verified in P. pastoris (Figure 4).

However, purification of the target proteins with a nickel column failed. McPGIP1 and McPGIP2 crude proteins were used to measure the inhibitory activity against PGs from A. niger using the DNS method. McPGIP1 and McPGIP1 inhibited PG activity levels from A. niger (Figure 5).

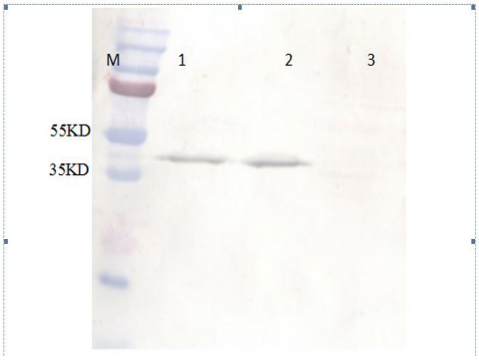

Figure 4: Western blot analysis showing target proteins. Lanes 1, 2 and 3 represent the supernatants containing McPGIP1, McPGIP2 and pPICZaA, respectively; single bands are visible for the supernatants containing McPGIP1 and McPGIP2, but there was no band from the supernatant containing pPICZaA, indicating that McPGIP1 and McPGIP2 are expressed in *P. pastoris.*

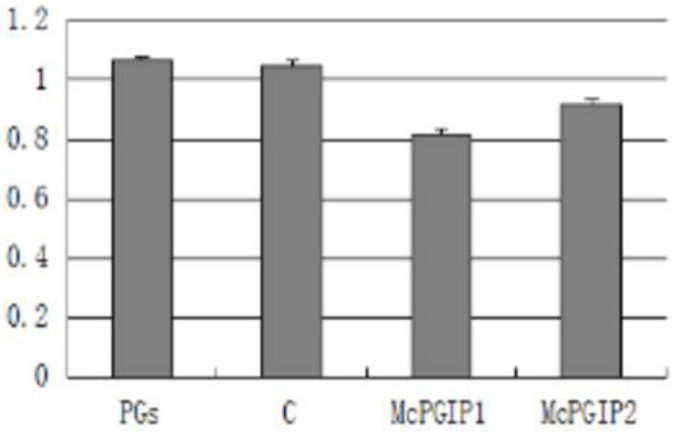

Figure 5: Inhibition of McPGIP1 and McPGIP2 to PGs of *A. niger.* McPGIP1 and McPGIP2 crude proteins were used to measure the inhibitory activity against PGs from *A. niger* using the DNS method. McPGIP1 and McPGIP1 inhibited PG activities from *A. Niger*

Discussion

Full-length cDNA

First, we used TAIL-PCR to harvest a full-length cDNA of Mcpgip1 and a partial sequence; we next used Fom-treated plants to extract total RNA, construct a cDNA library, design primers and rapidly amplify the cDNA ends using RACE-PCR. We also harvested another gene, Mcpgip2. The full-length cDNAs of Mcpgip1 and Mcpgi2 were 987 and 1005 bp, respectively, and no introns were included. However, we cannot be certain whether there are only two pgip genes in the plant. Different copy numbers of pgip genes were reported in different plants [7]. *P. vulgaris* has four PGIP genes that are induced in response to *S. sclerotiorum* infection [23], and various pgip genes can be introduced by different factors [1]. *B. napus* possesses a greatly expanded set of 16 or more pgip genes that are differentially expressed in response to wounding, defence hormones and *S. sclerotiorum* infection [15]. Thus, we cannot be sure that there are only two pgip genes in *M. charantia.*

Expression of PGIPs in Plant Organs

Plants were infected with Fom, and real-time quantitative PCR showed that PGIP expression increased after pathogen introduction. Weurman reported that PGIPs are extensively distributed in healthy plants, though at very low levels. Moreover, pathogens and stress can increase PGIP expression [24]. Our results also showed that different pathogens can result in differential expres-

sion, and the same PGIP can exhibit different expression profiles in different pathogens. These results confirmed those of Sathiyaraj, who used *Cylindrocarpon destructans, B. cinerea, Rhizoctonia solani, Colletotrichum gloeosporioides, Phythium ultimum* and *F. oxysporum* to infect Panax ginseng *C.A. Meyer*; the expression of PGIP infected by *C. destructans, B. cinerea, R. solani, C. gloeosporioides, P. ultimum, F. oxysporum* decreased [18]. Moreover, upon comparing the maximum relative expression and the time to maximum relative expression, we concluded that McPGIP2 may have a more important role in plants than McPGIP1 when pathogens are used as a method of introduction. Our results are supported by many other reports. For instance, Pvpgip3 responds to OGs but not to fungal glucan, salicylic acid or wounding; Pvpgip1 responds only to wounding, while Pvpgip2, which encodes the most efficient inhibitor of fungal PGs, is up-regulated by all of these stress stimuli [23].

Expression of *M. charantia* pgips in *P. Pastoris*

Mcpgip1 and Mcpgip2 were cloned into *P. pastoris*. Methyl alcohol was used to introduce the target protein. A hexahistidine tag was added to the target protein, and an anti-hexahistidine antibody recognized it in Western Blot analysis after the total proteins were separated by SDS-PAGE. SDS-PAGE and Western Blot demonstrated that the target protein was present in the supernatant. At the same time, DNS detected the crude activities of McPGIP1 and McPGIP2 against PGs from *A. niger*. We did not harvest the target proteins because the hexahistidine-tagged protein failed to bind to the affinity column. The same high levels of production and lack of harvested protein were observed by Hegedus when expressing BnPGIP1, BnPGIP2, BnPGIP7 and BnPGIP16 in *P. pastoris* [16] and by De Lorenzo when he intended to purify PvPGIP2 and GmPGIP1 [1]. Thus, we concluded that the target genes were expressed but that the tag was buried or hidden and could not be used to purify the protein.

Conclusion

We harvested two pgip genes, Mcpgip1 and Mcpgip2, in M. charantia for the first time. Real-time PCR indicated that FOM is an inducing factor, as McPGIP1 and McPGIP2 expression in the stem base increased. Under the same conditions, McPGIP2 expression was higher than that of McPGIP1. We concluded that McPGIP2 might have a more important role in plants than McPGIP1 when pathogens are introduced.

Mcpgip1 and Mcpgip2 were cloned into pPICZaA and expressed in *P. pastoris*. According to SDS-PAGE and Western Blot results, McPGIP1 and McPGIP2 are expressed in *P. pastoris*, but we failed to harvest pure McPGIP1 and McPGIP2. We measured the crude enzymatic activities of McPGIP1 and McPGIP2, which inhibited the activity of PGs from *A. niger*.

In summary, McPGIP1 and McPGIP2 from *M. charantia* can inhibit PG activity. PGIP has acquired the capacity to inhibit PG activity [2,3], but this report is the first describing the expression of PGIPs from *M. charantia* and contributes to our understanding of the PGIP family.

Author contributions

WLF detected the expression of *M. charantia* PGIPs in plant organs and in *P. pastoris*. The full-length cDNA was harvested by SYK. QSW provided great help during experiments. WZZ, LYF and JCY provided meaningful suggestions for the design of the paper.

Acknowledgements and funding

This work was supported by grants from the National Natural Science Foundation of China (NSFC, No. 30971888) and the Special Fund for Agro-scientific Research in the Public Interest from the Ministry of Agriculture (200903049-05).

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