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Real-time quantitative PCR analysis of expression pattern of a hypoglycemic polypeptide-P in *Momordica charantia* during plant growth

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Abstract: This study aimed to establish a real-time quantitative polymerase chain reaction (RT-qPCR) method to rapidly and reliably analyze hypoglycemic polypeptide-P gene expression of *Momordica charantia* (MC), and to examine the gene expression changes in different MC accessions, growth stages, and tissues harvested in different seasons. The RT-qPCR results were further verified by using Western Blotting (WB) analysis to compare the consistency between the polypeptide-P gene transcription and the corresponding protein expression. Nineteen accessions of MC were collected and tested in this study. Among the tested accessions, RU5H showed the highest expression level of polypeptide-P. The expression level of polypeptide-P was not only season-related, with the highest in the early July, but was also tissue-related, with the highest in the seed. In addition, the expression characteristic of polypeptide-P was maturity-related, with the highest expression level in the tender MC. The WB results showed that the transcription levels of this gene in different accessions displayed a more or less similar trend with the corresponding protein express.To sum up, the established RT-qPCR method can rapidly and effectively detect the expression level of polypeptide-P gene of MC, and various factors, including the accessions, harvesting seasons, maturity stages, and tissues can affect the expression level.

Keywords: *Momordica charantia*; Hyperglycemic polypeptide-P; RT-qPCR; Western Blotting; Express pattern

1. Introduction

Diabetes Mellitus (DM) is an endocrine disorder with the symptom of persistent high blood glucose which is caused by the decrease or defect of insulin[1, 2]. At present, DM has become the third killer following cardiovascular diseases and cancers because of unhealthy diet and lifestyle[1-3].

Generally, diabetics need to keep taking drug over their entire lifetime, hence medication and treatment of diabetes require a higher security standard. Therefore, it is of great importance to develop a novel diabetes drug with low side effects[4, 5].

Momordica charantia (MC) has been traditionally used for the treatment of diabetes in southeast Asia and China due to the antihyperglycemic and antioxidant activities[3, 6-9], and the remarkable

hypoglycemic function of MC has also been widely acknowledged[10, 11]. Accumulating evidence has demonstrated that MC polypeptide, as the main active ingredient, plays a critical role in hypoglycemic function[12-16]. Khanna and his peers has isolated some hypoglycemic polypeptides from certain India MC varieties, in which a water-soluble polypeptide component was named as polypeptide-P[14]. Many bio-active ingredients, such as some polypeptides, vary dramatically among different MC accessions, which can result in significant difference of antihypoglycemic activity[5, 17].

In our previous study, we have reported that the protein content of polypeptide-P exhibited distinct difference in various MC varieties[18]. Therefore, selecting suitable MC accessions with high hypoglycemic activity is important for patients with diabetes to relieve symptom and to reduce the medication costs. To select a high hypoglycemic active MC accession, the traditional method is to purify polypeptide-P protein from different MC varieties and then to compare the relative content of the purified protein[19]. Due to low content of polypeptide-P protein in plants, this screening process is usually time-consuming and laborious, and the accuracy is also a problem.

A real-time quantitative PCR (RT-qPCR) is a potential method for rapid detection of target gene expression level, and has been widely used in species identification[20-24]. In 2010, our research team has successfully cloned nucleotide sequence of polypeptide-P from MC[25]. In order to understand expression pattern of hypoglycemic polypeptide-P and to identify key factors affecting the express level in different MC varieties, we established a RT-qPCR procedure in this study to analyze mRNA level of MC polypeptide-P, and the corresponding protein expression level was also analyzed by western-blotting (WB) assay. Our results showed that the expression level of polypeptide-P in MC was affected by many factors, such as the accessions, growing seasons, fruit maturity, and tissues.

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2. Materials and Methods

2.1 Plant materials

Nineteen MC accessions were collected from different regions (Table 1). They were grown in natural environments. Mature fruits were respectively collected in the summer (June and July) and autumn (September and October) of 2015 and were immediately frozen at -80 ℃.

2.2 DNA extraction

Genomic DNA was extracted from MC by the improved CTAB (hexadecyl trimethyl ammonium bromide) method described by Porebski et al[26]. DNA was recovered by centrifugation, dissolved in TE (Tris 10 mM, EDTA 1 mM, pH 8), verified by using 1 % (wt/v)

agarose gel electrophoresis by comparison to the standard sample, and then quantified by U-2900 double beam spectrophotometer (Hitachi, Japan) at 260 nm. All DNA samples were diluted with sterile distilled water to obtain a final concentration of 20 μg mL^{-1}, and stored at -20 ℃.

2.3 RNA isolation and quality control

Total RNAs from the fruits and seeds were extracted using the TRIZOL reagent (TaKaRa, Dalian, China) by the modified guanidine hydrochloride method[27]. To remove contaminating DNA, the isolated RNA was treated with RNase-free DNase (TaKaRa, Dalian, China). Concentrations of each RNA sample were measured using U-2900 double beam spectrophotometer (Hitachi, Japan). Only the RNA samples with $OD_{260\ nm}/OD_{280\ nm}$ ratio (an indication of protein contamination) between 1.9 and 2.1 as well as $OD_{260\ nm}/OD_{230\ nm}$ ratio (an indication of reagent contamination) greater than 2.0 were used for the analysis. The integrity of RNA samples was also assessed by agarose gel electrophoresis. A total of 800 ng of total RNA were used to synthesize cDNA via the reverse transcriptase (TaKaRa, Dalian, China) following the protocol of manufacturer, and the cDNA sample was used as a template to quantify target gene expression level.

2.4 Protein isolation

A simple protocol was used for protein isolation from fresh fruits[28]. Briefly, equal mass of MC fruits was ground in liquid nitrogen to yield a fine powder. The powder was quickly transferred to a tube containing phosphate-buffered saline (PBS) (2:1, w/v). The mixture was stirred continuously in a mortar for 2 h at room temperature and was centrifuged at 6,000 × g for 15 min at 25 ℃. The supernatant was collected for further assay.

2.5 Real-time quantification PCR primer design

Two MC housekeeping genes (18S rRNA and β-actin) were selected as the candidate reference genes. The MC-specific 18S rRNA gene primer set MC-18SF/MC-18SR was designed based on the published MC 18S rRNA gene sequences (Table 2). The MC-specific β-actin gene primer set MC-actinF/MC-actinR was designed based on our cloned MC sequence (Table 2). The polypeptide-P gene specific primers were designed according to previously desribe[22]. The performance of the designed primers (Table 3) was measured by ~~the~~ melting curve analyses, and the quality was checked by agarose gel electrophoresis. We regarded the generation of only one single band of expected size and the single peak obtained from the melting curve as the criterion for specificity.

All the primer pairs were designed with the Primer 3 (version 0.4.0) program (http://frodo.wi.mit.edu). In a series of initial experiments, PCR product sizes were checked on a 1 % agarose gel prior to confirming the specificity of primers by sequencing. For each candidate reference gene, the PCR efficiency ~~of primers~~ was calculated using a real-time PCR on a standard curve generated using a 160-fold dilution series of a bulked complementary DNA (cDNA) sample over four dilution points which were measured in triplicate (Table 2).

2.6 Real-time quantification PCR analysis

Relative quantitative PCR expression analysis of selected genes was used in this study[29]. The RT-qPCR expressions of selected genes were performed with specific oligonucleotide primers (Table 3). The total RNA was estimated spectrophotometrically at 230, 260 and 280 nm (U-2900 double beam spectrophotometer), respectively. The same amount (800 ng) of total RNA was taken to synthesize the first strand cDNA using Superscript reverse transcriptase (TaKaRa, Dalian, China). The reactions were performed using SYBR Green detection chemistry and run in triplicate on 96-well plates with the StepOnePlus™ (Applied Biosystems, US). Reactions were prepared in a total

volume of 20 µl containing 4 µl of template, 2 µl of each amplification primer, 10 µl of 2 × FastStart SYBR Green Master (TaKaRa, Dalian, China), and 2 µl of fluorescein as normalization dye. Blank controls were run in triplicate for each master mix. The cycling conditions were set as follows: initial denaturation step of 95 ℃ for 10 min to activate the FastStart Taq DNA polymerase, followed by 45 cycles of denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 30 s and extension at 72 ℃ for 30 s. Baseline and threshold cycles (Ct) were automatically determined using the StepOne™ System .RT-qPCR analyses were carried out with three independent total RNA samples. Percent of gene expression was determined by normalizing values against internal control β-actin. Values were represented as percent of gene expression with respect to corresponding controls ~~and plotted using Microsoft Excel.~~

2.7 Western blot assay

For WB assay, a total of 100 ng protein, detected by using the colloidal Coomassie Brilliant Blue (CBB) (Beyotime, Nanjing, China) method, were combined with an equal volume of 2 × SDS-PAGE loading buffer and then heated to 100 ℃ for 8 min and separated by 13.5 % Tris-Tricine SDS-PAGE. Proteins were electroblotted onto a PVDF membrane at 100 V for 90 min using a wet blotting system (Pall Life Sciences, New York, USA). The electroblotted membranes were blocked with 5 % skim milk in PBS containing 0.5 % Tween-20 (PBS-T buffer) overnight at 4 ℃. After washing the membranes three times with PBS-T buffer, an anti-polypeptide-P antibody[18], diluted 1:2,000 in PBS-T buffer, was added and incubated for 8 h at 4 ℃. Afterwards, the membranes were washed three times using PBS-T buffer. Next, the membranes were incubated with an anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase (Biotium, Hayward, CA, USA) at a dilution of 1:2,000 in PBS-T containing 1 % skim milk for 2 h at room temperature. The signal was detected by DAB chemical chromogenic reagents (Beyotime), according to the instructions of manufacturer.

2.8 Statistical analysis

Changes in the relative expression levels (REU) of gene mRNA were checked for statistical significance according to Student's t-test. The results were considered statistically significant if the P-value was < 0.05. The WB results were analyzed using Image J 2x and SPSS 19.0 software. The Microsoft excel file of raw expression values for the tested genes in 10 different samples was given in Table 1.

Table 1. List of *Momordica charantia* accessions, origins and fruit character

No.	Accession	Origin [a]	Fruit character [b]
1	DB	Hunan	White, medium long, continuous ridges
2	CB	Hunan	Light green, medium-long [d], continuous ridges
3	CFLP	Vietnam	Dark green, small [c], continuous ridges
4	HLLY	Sichuan	Light green, medium-long, continuous ridges
5	LP	Hunan	Green, long [e], discontinuous ridges
6	YYEH	Shandong	Dark green, small, continuous ridges
7	HYXC	Hebei	Light green, medium-long, discontinuous ridges
8	GDQP	Hunan	Glossy green, long, continuous ridges
9	RU5H	Fujian	Glossy green, medium-long, discontinuous ridges
10	TDCB	Hunan	Greenish white, extra-long [f], discontinuous ridges
11	TWTC	Taiwan	Glossy green, extra-long, discontinuous ridges
12	CL	Guangdong	Light green, long, continuous ridges
13	QP	Hebei	Glossy green, medium-long, continuous ridges
14	LMKG	Sichuan	White, extra-long, continuous ridges
15	XC2H	Fujian	Green, medium-long, continuous ridges
16	JGDR	Changsha	Glossy green, medium-long, discontinuous ridges
17	TCT	Guangdong	Glossy green, long, discontinuous ridges

18	DDCL	Hubei	Glossy green, medium-long, discontinuous ridges
19	CQKG	Vietnam	Green, small, continuous ridges

[a] Country or Chinese province from where the accession was collected. [b] Fruit color, size and surface texture. [c] Small (<10 cm). [d] Medium-long (10 - 20 cm). [e] Long (20 - 30 cm). [f] Extra-long (>30 cm).

3. Results

3.1 Real-time PCR primer selection of reference gene

Real-time PCR method has been widely used for gene expression analysis. The housekeeping genes, 18S rRNA and β-actin, have been widely applied in real-time PCR analysis and often used as candidate genes for internal control. The MC 18S rRNA sequence obtained from GenBank was identified by using PCR amplification and sequencing analysis (data not shown). Because there is no published MC β-actin gene sequence in GenBank, the partial MC β-actin nucleotide sequence was cloned by designing a pair of degenerate primers based on the alignment result of available cucurbitaceae β-actin gene sequences. The gene alignment results of β-actin from three cucurbitaceae plants (Cucumis melo, Cucumis sativus and Citrullus lanatus) displayed high identity (Figure 1). Therefore, a pair of primers were designed to amplify this high conserve region of β-actin gene. The amplified DNA fragment was recovered and sequenced, which showed high similarity to the β-actin gene sequences of Cucumis melo (96%, AB640865.1), Cucumis sativus (95%, AB010922.1), and Citrullus lanatus (94%, GU565958.1) (Figure 1). Additionally, ~~the~~ new primers were designed according to the cloned DNA sequence information and used as internal control for RT-qPCR analysis (Table 2).

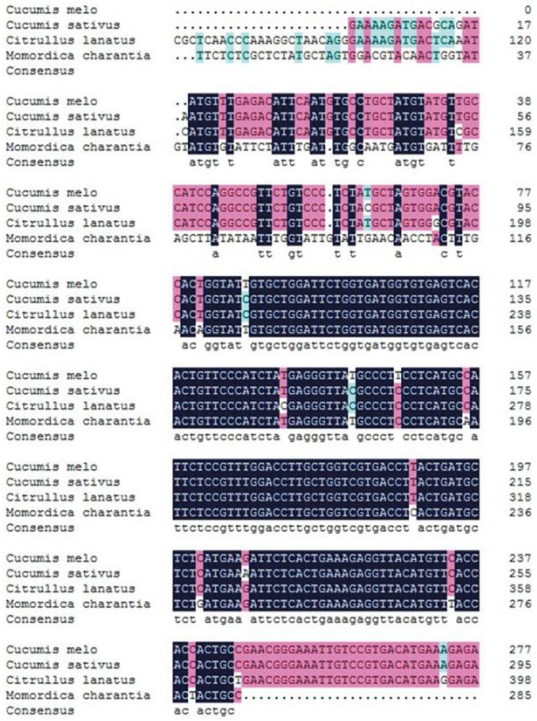


Figure 1. Sequence analysis of β-actin from different ~~kinds of~~ cucurbitaceae including *Momordica charantia*. Alignment of the nucleotide sequences of cucurbitaceae from *Cucumis melo* (AB640865.1), *Cucumis sativus*

(AB010922.1), *Citrullus lanatus* (GU565958.1) and the amplified DNA fragment from MC. The ~~four~~ highly conserved motifs are indicated by the blue Characters background.

Table 2. Reference gene and designed primer set used for real-time PCR analysis

Gene	Accession No	Gene description	Primer name	Amplified length (bp)	E%	R^2
18S rRNA	JN407450.1	MC 18S ribosomal RNA	MC-18SF MC-18SR	232	1.086	0.991
β-actin	AB640865.1 AB010922.1 GU565958.1	*Cucumis melo* *Cucumis sativus* *Citrullus lanatus*	MC-actinF MC-actinR	124	1.161	0.997
ssdp	HQ164449.1	MC polypeptide-P	MC-ssdpF MC-ssdpR	148	1.174	0.999

* The RT-qPCR efficiency and correlation coefficients (R^2) were determined with LinRegPCR software.[28, 29]

The total RNAs of 11 accessions of MC were extracted and then reverse-transcribed. After 40-cycle-amplification by using β-actin or 18S rRNA primers respectively, the melting-curves were almost identical among these 11 accessions, suggesting similar expression level of β-actin (Figure 2A) or 18S rRNA (Supplemental Figure 1) in different accessions of MC. The agarose gel electrophoresis confirmed that the PCR products of 11 accessions were of equal brightness at 241 bp (Figure 2B). In brief, these findings demonstrated that the expressions of both β-actin and 18S rRNA in MC were stable regardless of accession difference.

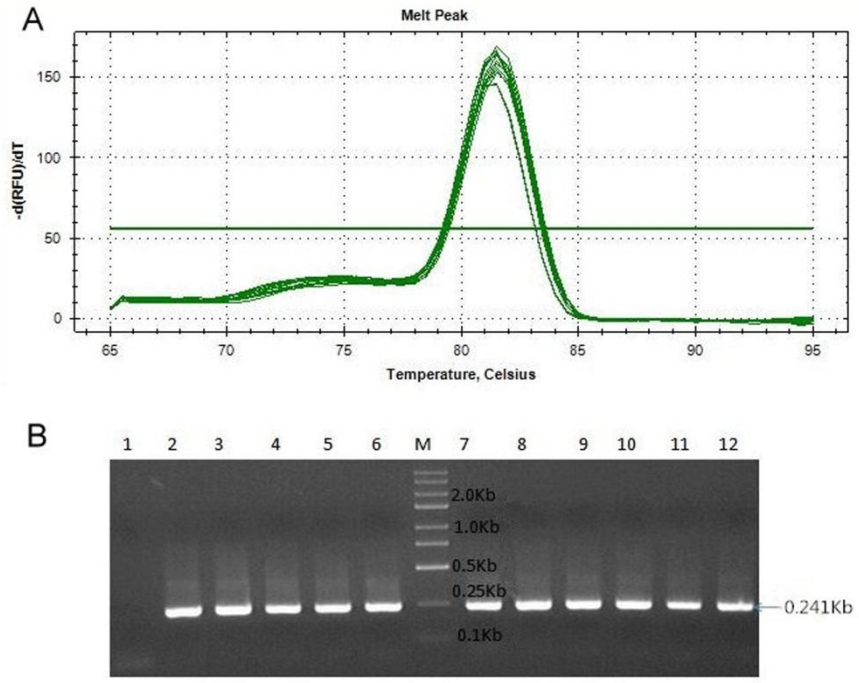


Figure 2. Specificity of real-time PCR amplification. (A) Melt curves for β-actin refer gene with single peak obtained from three technical replicates of 11 different cDNA of MC. Melt curves for 18S rRNA genes with single peak are shown in Supplemental Figure 1. (B) Agarose gel (2%) showing amplification of a specific PCR product of expected size for each MC accessions tested in the study. M: Maker; 1: no template control (NTC); 2-12: CFLP; HLLY; YYEH; GDQP; DDCL; LMKG; CQKG; QP; FLYH; YEEH; CLKG

Based on the expression proximity between internal control gene and target gene, the data (Figure 3) suggested that β-actin might be the most suitable housekeeping gene to be used in further study. Of note, in terms of the expression profiling in the MC samples, β-actin (Average CT value ranging from 22 to 26) showed closer level of expression to polypeptide-P (average CT value ranging from 27 to 34) and was therefore chosen for further study.

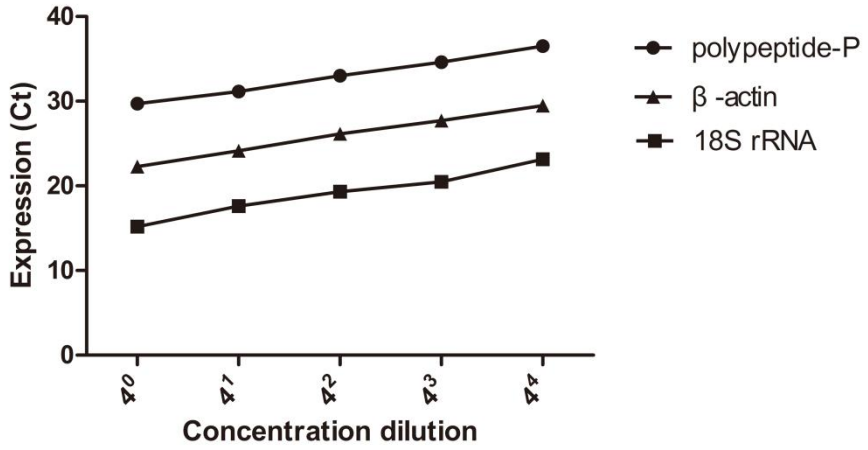


Figure 3. Expression stability and the CT value of the target-gene and candidate reference gene as measured by real-time PCR in five concentration dilution. The expression of β-actin (Average CT value ranging from 22 to 26) was closer with the expression of polypeptide-P (average CT value ranging from 27 to 34).

3.1 Real-time PCR primer selection of polypeptide-P

A total of 5 primer pairs were selected as candidates for detection of polypeptide-P gene expression (Table 3). The performance of the amplification primers was tested by real-time PCR in two ways. First, aliquots from the 8 cDNA samples were pooled and used as a template in amplification reaction with each primer pair. As summarized in Table 4, only MC-SSDPF/MC-SSDPR primer pair obtained from MC has a single peak (Figure 4A) in melt curves sufficiently different from those of corresponding amplicons to allow detection of primer-dimers interference in a homogeneous assay (date not show). And the standard agarose gel electrophoresis were followed by a real-time RT-PCR. A single band with the expected size (Table 2) was obtained in each case without signs of primer-dimers formation (Figure 4B), as suggested by the previous melting curve analyses (Figure 4A). Thus, MC-SSDPF/MC-SSDPR was chosen as the target-gene primer pair.

Table 3. Polypeptide-P gene and designed prime set used for real-time PCR analysis

Accession No	Primer name	Sequence	Amplified length (bp)
HQ164449.1	MC-SSD1F	5'GATCGTGGCGCCGTCTAC-3'	106
	MC-SSD1R	5'GGTTCATGGATAGAACGTTTCACTT-3'	
	MC-SSD2F	5'CTATGACCGATGGCAAAGATGAT-3'	87
	MC-SSD2R	5'AGACGCGCCAAAATCTTTTTC-3'	
	MC-SSD3F	5'CGTGATGAAGGCAAAGTGGAT-3'	114
	MC-SSD3R	5'AGACGCGCCAAAATCTTTTTC-3'	

MC-SP1F MC-XSP1R	5'AGTGGATGAATCTATGACCGATGGC-3' 5'CGCATGTTTCAGTTCCGCAGATTCCG-3'	127
MC-SSDPF MC-SSDPR	5' CGTGATGAAGGCAAAGTGGA-3' 5' TATCGCCAAACGGGGTAATG-3'	148

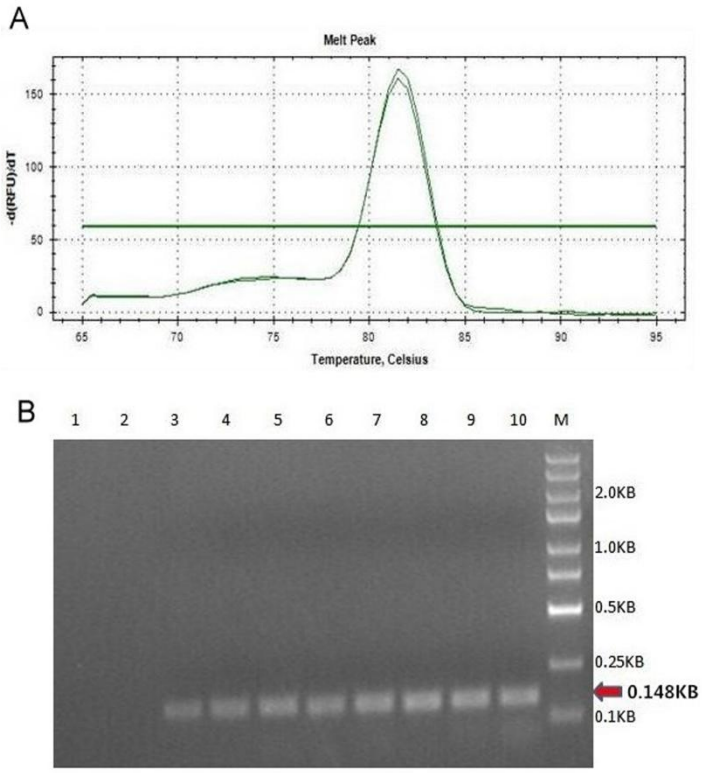


Figure 4. MC-SSDPF/MC-SSDPR of real-time PCR amplification? (A) Melt curves for SSDP with single peak obtained from three technical replicates of MC Melt curves. (B) Agarose gel (2 %) showing amplification of a specific PCR product of expected size for each MC accessions. M: Marker; 1, 2: no template control (NTC); 3-10: CFLP; HLLY; YYEH; GDQP; DDCL; LMKG; CQKG; QP.

3.2 Expression analysis of polypeptide-P gene in different accessions

we performed relative-quantitative RT-PCR to analyze the expression levels of polypeptide-P in 10 different MC accessions, based on the diverse apparent classes and origin and occurrence. In order to control gDNA contamination in the cDNA samples, we examined the cDNA samples before the test. PCR primers were designed on different exons (Table 4) and tested by PCR. On the one hand, amplification primers were tested using gDNA and cDNA as templates, respectively (Figure 5A). Beta-actin primer-pair yielded amplicons longer than those obtained with a cDNA template. On the other hand, aliquots from the 4 cDNA samples were pooled and used as template in amplification reactions with β-actin primer-pair. A single band with the expected size (Table 4) was obtained in two cases without signs of primer-dimers formation (Figure 5B, lanes 4, 5) and the other PCR products had two bands on gel, which implied that the cDNAs of lanes 2, 3 were interfused with genome. This result implied that intron position prediction in MC β -actin genes was successful and we could examine the genomic contamination of cDNA samples.

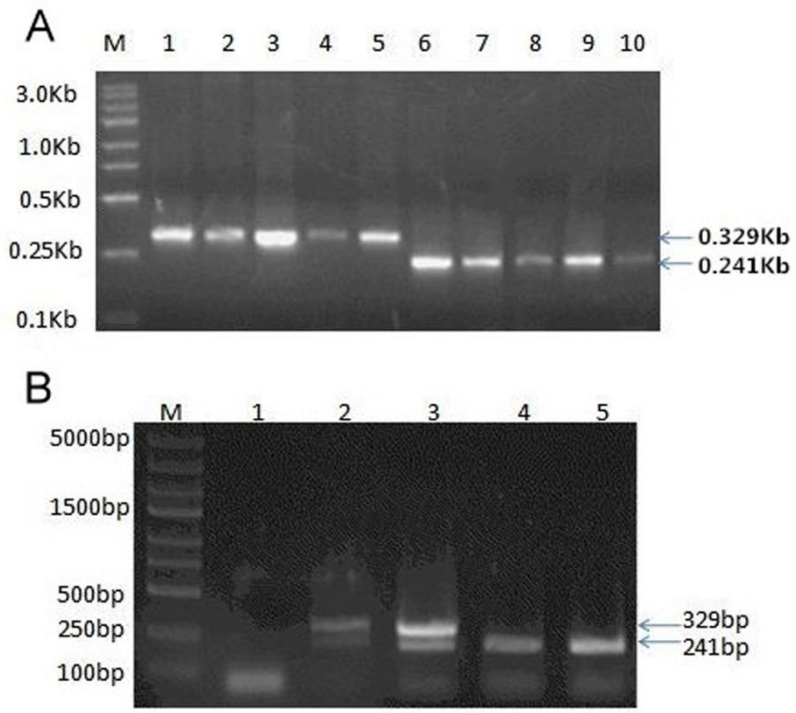


Figure 5. Electrophoresis diagram of PCR product of β-actin. (A) Electrophoresis diagram of PCR product of β-actin at DNA and cDNA level. M: Maker; 1-5: CFLP; HLLY; YYEH; GDQP; DDCL; Agarose gel electrophoresis of PCR products in DNA level. 6-10: CFLP; HLLY; YYEH; GDQP; DDCL; Electrophoresis of PCR products in cDNA level. (B) Electrophoresis diagram of PCR product of β-actin at cDNA level. M: Maker; 1: blank control; 2-3: Electrophoresis diagram of cDNA with genomic contamination; 4-5: Electrophoresis diagram of cDNA without genomic contamination.

Table 4. Reference gene prime set used for real-time PCR analysis

Primer name	Sequence
MC-18SF	5'-CGTAGCGAAATGCGATACTTGGTGTG-3'
MC-18SR	5'-CAGGCGCCGAGGACTCGGATTTAAG-3'
MC-actinF	5'-CCCTCCCTCATGCAATTCTC-3'
MC-actinR	5'-TCGGCAGTAGTGGTAAACATGTAAC-3'
MC-ssdpF	5' CGTGATGAAGGCAAAGTGGA-3'
MC-ssdpR	5' TATCGCCAAACGGGGTAATG-3'

The RT-qPCR results (Figure 6A) showed that the analysis of the gene-expression which suggested the mRNA levels of polypeptide-P in LP and DB were higher (49 % and 48 %, respectively) than that of the control (CB). However, the expression levels of polypeptide-P in CL, TDCB, TWDC and XC2H were much lower (decreased about 19 %-92 %). And relative mRNA level of polypeptide-P in RU5H was 2.8 times higher than that of the control. These data indicated that the expression of polypeptide-P varied among different accessions of MC, and RU5H displayed the highest polypeptide-P expression, while XC2H had the lowest.

Furthermore, WB analysis was carried out to examine the expression of polypeptide-P in different MC accessions (Figure 6B). The results suggested that the polypeptide-P protein expession levels in LP, DB and RU5H were relatively high, however, the protein expression in CL, TDCB, TWDC and XC2H was much lower, which was consistent with the results in Figure 6A, suggesting that RT-qPCR was an reliable and efficient way to detect the polypeptide-P expression level.

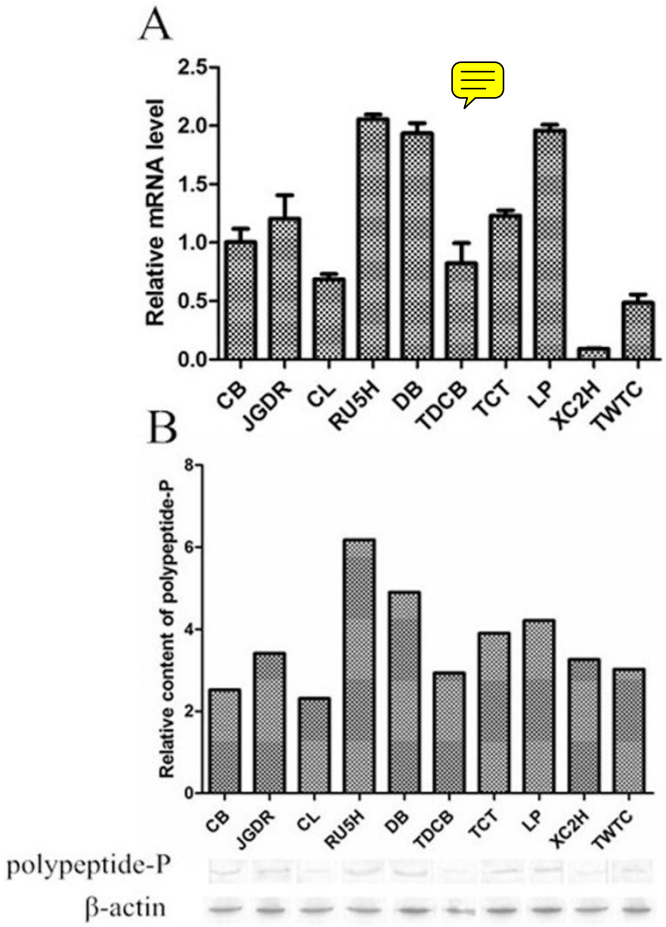


Figure 6. Differential transcription of the polypeptide-P gene from *Momordica charantia* in real-time PCR analysis (A) and western blot analysis (B). (A) Total RNAs were isolated from different accessions of MC and 1 mg of total RNA was used for the real-time PCR assay. The cDNA fragments were amplified by PCR reaction with primers for the polypeptide-P gene and β-actin. Bars above each lane represent the results of content of respective polypeptide-P bands using real-time PCR, given as folds of the CB accession (control). (B) Polypeptide-P from 10 *Momordica charantia* accessions was detected by western blot with anti-polypeptide antibody. The upper bands are consistent with the transcription patterns of the polypeptide-P gene and the lower band corresponds to transcripts from the β-actin gene (internal control). Bars above each lane represent the results of content of respective polypeptide-P bands using Image J software, given as folds of the CB accession (control).

3.3 Expression analysis of polypeptide-P gene during different harvesting time

At different harvesting seasons, the gene expression levels of polypeptide-P were different. Figure 7A indicated that the expression level in RU5H was at the highest on the July 6th, and then declined gradually during the July but droped dramatically after the August. As to JGDR, TDCB,

and DB, there also had an initial expression peak of polypeptide-P on the July 6th, but had little expression after that date. These data indicated that the expression of polypeptide-P was time- or season-related, and tended to have a relative higher expression level during early July in which the MC fruit is mostly immature. To testify this hypothesis, we compared mature fruit with immature fruit about the polypeptide-P expression levels. Figure 7B showed that among four tested accessions of MC, all immature fruit expressed more polypeptide-P than the mature fruit, indicating that the polypeptide-P transcript levels decreased with the maturity and development of fruit.

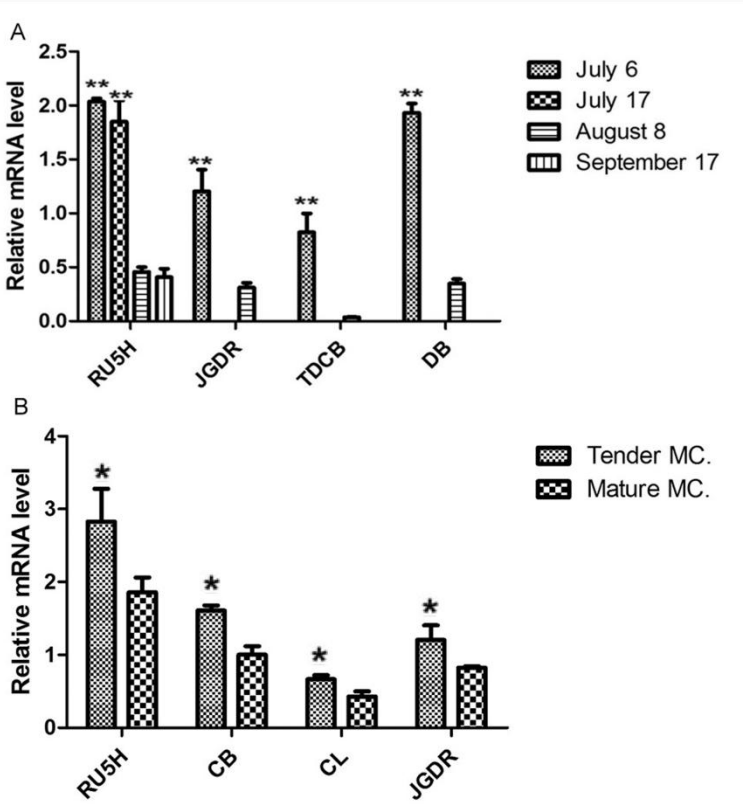


Figure 7. Relative-quantitative RT-PCR analysis of expression of four different species in different harvest time and maturity. (A) Expression of the polypeptide-P gene in different harvest time picked on July 6, July 17, August 8 and September 17. Total RNAs were isolated from different kinds of MC. picked in different time, and 1 mg of total RNA was used for real-time PCR. The cDNA fragments were amplified by PCR reaction with primers for the polypeptide-P gene and β-actin. The transcript levels of the polypeptide-P at each species were plotted as percentages of the control ((CB). (B) Expression of the polypeptide-P gene upon different growth status. The transcript levels of the polypeptide-P at each species were plotted as percentages of the control. All of the presented data are averages and standard errors (bars) of three independent experiments. The double asterisks indicate that the treatment was significantly different from the control at $P < 0.01$. The single asterisk indicates that the treatment was different from the control at $P < 0.05$.

3.4 Expression analysis of polypeptide-P gene in different tissues

Furthermore, we examined the polypeptide expression levels in different tissues. The results in Figure 8 showed that the expression pattern of polypeptide-P wa[illegible]anscriptionally active in various tissues, but their transcriptional levels differed. Transcription accumulated in leaf, fruit and seed of MC. Much lower levels of polypeptide-P transcription were observed in stem and flower

than in developing seed and fruit (Figure 8). In particular, relatively high level of polypeptide-P transcription was found in developing seed. Thus polypeptide-P showed a spatially differential expression pattern in MC.

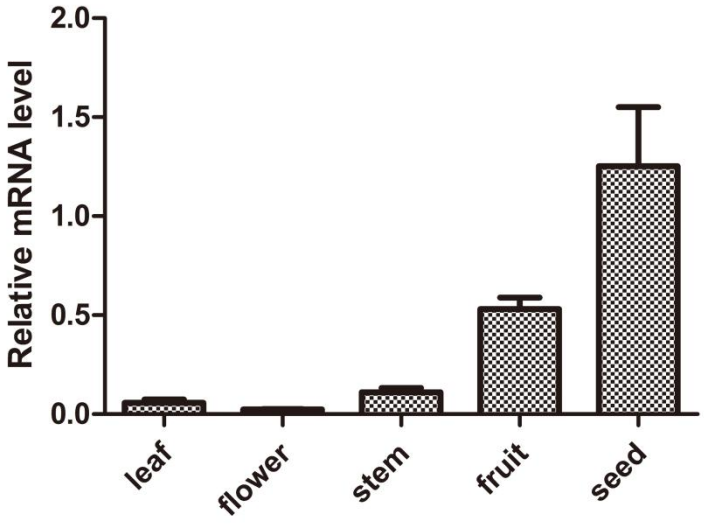


Figure 8. Expression of the polypeptide-P gene in different tissues of *Momordica charantia*. (Given as relative yield of mRNA level normalized to CB accession). Total RNAs were isolated from different tissues of MC and 1 ng of total RNA was used for the real-time PCR assay. The cDNA fragments were amplified by PCR reaction with primers for the polypeptide-P gene and β-actin. Bars above each lane represent the results of content of respective polypeptide-P bands using real-time PCR.

4. Conclusions

In summary, the RT-qPCR method described in the study provided a rapid and universal assay system for detecting hypoglycemic polypeptide-P in MC. We selected MC-specific β-actin gene primer set MC-actinF/MC-actinR as internal control and polypeptide-P gene primer MC-SSDPF/MC-SSDPR was designed as RT-PCR primer based on previously cloned MC polypeptide-P sequence. Also, we identified the polypeptide-P gene in MC and studied its expression pattern. More importantly, these findings showed that the polypeptide-P gene was highly expressed in the RU5H and DB compared with other accessions, which was consistent with the result of WB analysis. In addition, the expression pattern of polypeptide-P was time- and tissue-related, and the highest expression level was found during early July, indicating an early regulation of the gene in response to the season. And the polypeptide-P transcription accumulated in leaf, seed, and fruit, especially immature fruit. The present study, therefore, revealed the distribution of polypeptide-P and provided a reliable reference for cultivating and using high quality hypoglycemic MC.

Supplementary Materials: Figure S1. Specificity of real-time PCR amplification for 18S rRNA. (A) Melt curves for 18S rRNA refer gene with single peak obtained from three technical replicates of 11 different cDNA of MC. (B) Agarose gel (2%) showing amplification of a specific PCR product of expected size for each MC accessions tested in the study. M: Maker; 1: no template control (NTC); 2-12: CFLP; HLLY; YYEH; GDQP; DDCL; LMKG; CQKG; QP; FLYH; YEEH; CLKG

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Project administration, Zhao Jian; Visualization, Wang Yi-Shuai and Li Peng-Fei; Writing – original draft, Wang Yi-Shuai; Writing – review & editing, Wang Fu-Jun and Zhao Jian.

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