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*Full Length Research Paper*

# Molecular cloning and identification of tissue-specific expression of ent-kaurene oxidase gene in *Momordica charantia*

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***Momordica charantia*** **(bitter melon) is widely consumed as a vegetable and as a folk medicine. Ent-kaurene oxidase is a key enzyme of gibberellin (GA) synthesis by controlling the early GA biosynthesis. In this study, ent-kaurene oxidase cDNA sequence was successfully amplified from the total cDNA of pistillate flower buds, and a complete open reading frame (ORF) with 1563 bp, encoding 520 amino acids, containing 151 bp of 5'-untranslated region sequences and 217 bp of the 3'-untranslated region sequences was found. Quantitative real-time polymerase chain reaction (qRT-PCR) revealed that the expression level of *M. charantia* ent-kaurene oxidase (*McEKO*) gene in pistillate flowers was significantly higher than in staminate flowers and neutral flowers, and the expression level was especially abundant in root and stem apexes. The gene was express in *Escherichia coli* cells with a molecular weight of 58.8 kD observed.**

**Key words:** Gibberellin, bioinformatics analysis, procaryotic expression, quantitative real-time polymerase chain reaction qRT-(PCR).

## INTRODUCTION

Gibberellins (GA) are a bewilderingly large group of diterpenoid plant hormone which participates in most, if not all, life cycles of plants. It cannot only promote the setting rate of plants, increase production, improve the drought resistance of plants such as resilience, but also promote the elongation of plant. GA controls higher plants through a species-specific, tissue-specific and develop-mental-specific biosynthesis (Lange and Lange, 2006). The GA biosynthetic pathway can be conveniently divided into three parts: (i) the synthesis of ent-kaurene from geranyl geranyl diphosphate by copalyl diphosphate synthase and ent-kaurene synthase activities; (ii) the conversion of ent-kaurene to GA53 by cytochrome P450 enzymes; and (iii) the further metabolism to biologically active GAs by dioxygenases (Helliwell et al., 1998). Ent-kaurene oxidase (EKO) plays a crucial role in GA biosynthesis pathway by catalyzing the three-step oxidation of ent-kaurene to ent-kaurene acid. *GA3* gene encodes ent-kaurene oxidase in *Arabidopsis*. The *ga3* mutant plant which is gibberellin-responsive dwarf was found deficient in ent-kaurene oxidase activity (Helliwell et al., 1999). *LH* gene encodes ent-kaurene oxidase in pea (*Pisum sativum*). The *lh* mutants are GA deficient and have reduced internode elongation and root growth or seed abortion (Davidson et al., 2004). In higher plants, several genes encoding KO have been functionally identified, such as in rice (Ko et al., 2008), strawberry (Shi et al., 2006) and pear (Li et al., 2010).

As a nutritious traditional vegetable in China and Southeast Asian countries, bitter melon (*Momordica charantia L*) is also used as an important medicinal plant for anti-diabetic, anticancer, antivirus and cholesterol lowering agent. The aqueous and chloroform extracts of *M. charantia* significantly potentiate glucose uptake and phytochemical compounds such as charantin, steroid, glycosides, flavonoid and their derivatives may in part have been responsible for the observed hypoglycaemic activity (Ramadhar et al., 2009). Glucose uptake assays on cells and in male mice suggested hypoglycaemic

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activities of methanol extraction of bitter melon fruits and active constituents were cucurbitane-type triterpenes (Chang et al., 2011). The anticarcinogenic effect of *M. charantia* was studied in a two-step skin carcinogenesis model in mice, which suggested that there is a preventive role of water-soluble constituents of bitter melon fruit (Ganguly et al., 2000) and the maximum chemo-preventive potential is in the bitter melon peel (Singh et al., 1998). Polyethylene glycol (PEG) modification of RIP might be potentially developed as anti-cancer drug (Li et al., 2009). Bitter melon extract modulates signal transduction pathways for inhibition of breast cancer cell growth and can be used as a dietary supplement for prevention of breast cancer (Ray et al., 2010). Data based on cell analysis and rats supported that bitter melon fruit extract possesses anti-oxidant effects (Budrat and Shotipruk, 2008; Kumar et al., 2010; Semiz and Sen, 2007). Momordica Anti-HIV Protein, α- and β-momorcharins inhibit HIV replication in acutely and chronically infected cells and thus, are considered potential therapeutic agent in HIV infection and AIDS (Puri et al., 2009). Bitter melon juice was proved to be potent inhibitor of lipogenesis and stimulator of lipolysis activity in human adipocytes (Nerurkar et al., 2010). In addition, aqueous extract of the green bitter melon fruits can be used against kalaazar caused by *Leishmania donovani* (Gupta et al., 2010).

In bitter melon, one of the major factors limiting quantity of fruit production is low pisillate flowers proportion, which may be as little as 12 to 16%. Previous studies showed that *in vitro* GA3 application during seeds germination and spraying of field grown plants both induced significantly higher number of pistillate flowers in bitter melon (Thomas, 2008). However, scientific information on regulation of GAs biosynthesis of bitter melon and genes coding for key enzymes in the pathway have hitherto been lacking. This study addresses this deficiency by cloning and characterizing expression patterns of ent-kaurene oxidase gene of *M. charantia* (*McEKO*). It is this basis that modifies sex expression in *M. charantia* on the level of gene engineering hence creates a chance for increasing fruit production.

## MATERIALS AND METHODS

*M. charantia* was obtained from Medicinal Botanical Garden of School of Life Sciences, Sichuan University, Chengdu, China. The top10 and BL21 of *Escherichia coli* and pET28a (+) vector were preserved in our laboratory.

### Isolation of RNA

Total RNA from female flower buds of bitter melon was extracted using plant RNA isolation reagent following the manufacturer's instructions (TaKaRa, Dalian,China). After treatment with DNaseI (Takara, Dalian, China) to remove contaminant genomic DNA traces, RNA quantity, purity and integrity were verified by electrophoresis on 1% agarose gel (Li and Wang, 1999).

### Cloning of core cDNA fragment

The cDNA was synthesized using the first-strand cDNA synthesis kit, (TaKaRa, Dalian, China) and oligo (dT) primer (Invitrogen, Shanghai, China). A pair of oligonucleotide primers GA3A and GA3B (Table 1) (Invitrogen, Shanghai, China) used for the cloning of *McEKO* core cDNA fragment were designed according to the conserved regions of ent-Kaurene oxidase gene of plants such as *Cucurbita maxima* (GenBank accession no. AF212991) (Helliwell et al., 2000), *Arabidopsis thaliana* (GenaBank accession no. 047719) (Helliwell et al., 1998) and *Pisum sativum* (GnenBank accession no. 245442) from GenBank.

Core cDNA fragment of *McEKO* gene was cloned according to the protocol of ExScrip RT-PCR Kit (TaKaRa). The amplified product was cloned into the pMD19-T vector and sequenced (Invitrogen, Shanghai, China). This fragment was subsequently used for designing specific primers for cloning of the 5' and 3' ends of cDNA of *McEKO* by RACE.

### Generation of the full-length cDNA

Nucleotide sequences of the 3' and 5' ends of cDNA of *McEKO* were amplified by rapid amplification of cDNA ends (RACE) (Sambrook and Russell, 2001) using four universal primers: AP1, AP2, oligo(dT)11AP, T7 and five gene-specific primers EKO31, EKO32, EKO51, EKO52, EKO53. The cDNA for 3' and 5'RACE were synthesized respectively by oligo (dT) primer and EKO51. PCR products were cloned into pMD19-T vectors (Takara, Dalian, China) and sequenced (Invitrogen, Shanghai, China).

EKF3 and EKF5, designed according to sequences of 3' and 5' RACE products, were used to amplify the full-length cDNA of *McEKO*. The full length cDNA was cloned into pMD19-T vectors and sequenced. All primers are listed in Table 1.

### Sequence analysis

The nucleotide sequence and ORF were analyzed by DNAMAN6.0. Structural analysis of deduced protein was carried out online at the websites (http://cn.expasy.org/tools/ and http://npsa-pbil.ibcp.fr) and by software of BioEdit7.0. The sequence comparison was conducted through database search using BLAST program (http://www.ncbi.nlm.nih.gov). A phylogenetic tree was constructed using Clustalx 1.8.

### Expression profile by quantitative real-time PCR analysis

Total RNA isolated from different tissues including stem apex, root apex, leaf, male flower, female flower, neutral flower and young fruit were synthesized into cDNA with ExScript$^{TM}$ RTase (Takara, Dalian, China). *M. charantia* 18S ribosomal RNA (18S rRNA) was used as an internal control to normalize the amount of mRNA present in each sample by primers MC18SRT1 and MC18SRT2 (Peng et al., 2006). *McEKO* gene-specific primers EKRT1 and EKRT2 were designed according to the full-length sequence of *McEKO* (Table 1). qRT- PCR reactions were performed with iCycler iQ$^{TM}$ real-time cycler (Bio-RAD. USA) using SYBR Premix Ex Taq$^{TM}$ (Takara, Dalian, China) and the cycling conditions of 95°C for 10 min, and 45 cycles of 95°C for 15 s, 58°C for 15 s and 72°C for 30 s, then 95°C for 1 min, and 55°C for 1 min. The products of qRT-PCR were confirmed by determining the melt curves for the products at the end of each run. LightCycler® 480 Software (version 1.5; Roche Diagnostics) was used to analyze the data and each data represents the average of three independent experiments.

**Table 1.** Primers used in *McEKO* cloning and expression analysis.

| Primer designation | Sequence(5'to3') |
|---|---|
| GA3A | GA(C/T)TGG(A/C)G(A/G/C/T)GA(C/T)TT(C/T)TT(C/T)CC |
| GA3B | C(G/T)(A/T)AG(A/G/C/T)GT(C/T)TC(G/C)TG(A/G)AA(A/G/C/T)AC |
| AP1 | GTCAACGATACGCTACGTAACG |
| AP2 | TACGTAACGGCATGACAGTG |
| oligo(dT)11AP | ACGACTCACTATAGGGCTTTTTTTTTTTMN |
| T7 | GTAATACGACTCACTATAGGGC |
| EKO31 | GAGATGGATTCCCAACAAGG |
| EKO32 | CCTCGGACACTACTTTGGTT |
| EKO51 | TCCTCAATAAGGCTCTTCATC |
| EKO52 | AAGGCTCTTCATCGTCACTGG |
| EKO53 | CCTTGTTGGGAATCCATCTC |
| EKF5 | TTTCGTCTTTCTTATCTTCGTCTTC |
| EKF3 | ACGACGACGAAACGCTTAC |
| MC18SRT1 | TGCCCGTTGCTCTGATGATTC |
| MC18SRT2 | CTGCTGCCTTCCTTGGATGTG |
| EKRT1 | ACCGGCGATCACTTCTGATA |
| EKRT2 | GATCCTTAGTTGGTTCTTGGACTA |

**Expression of *McEKO* in *E. coli***

The forward primer was designed to contain the BamHI site and the reverse primer to contain the NotI site. Amplified products were cloned into pMD19-T vectors (Takara, Dalian, China) for sequencing. The insert was digested with BamHI and NotI and gel was purified with the Gel Extraction Kit (Axygen scientific Inc., USA), then integrated into the pET28a (+) vector digested with the same enzymes. The recombinant pET28-EKO and pET28-empty plasmids were respectively transformed into *E. coli* BL21. Transformants were inoculated onto fresh medium containing antibiotics until the optical density (OD600) reached 0.6 after cultured in Luria–Bertani (LB) liquid medium containing 50 µg/ml kanamycin overnight at 37°C in a shaking incubator. Then, gene expression was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) into Luria–Bertani (LB) liquid medium to a final concentration of 1.0 mM, which is the appropriate concentration in preliminary experiment, and further incubated at 37°C for hours.

The cells were harvested by centrifuging at 4000 rpm for 20 min, then dissolved in 1×SDS sample buffer and boiled at 100°C for 5 min. After centrifuging at 10000 rpm for 1 min, the supernatant was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). The gel was stained for more than 4 h in Coomassie brilliant blue R250 methanol-acetic acid solution, and then bleached to obtain clear protein bands.

## RESULTS

### The full-length cDNA and sequence characterization of *McEKO*

A cDNA fragment of 413 bp was initially isolated with degenerate primers by the RT-PCR method. Nucleotide BLAST search showed that the core fragment shared high homology with many known ent-kaurene oxidase genes, indicating that the cDNA fragment was isolated from ent-kaurene oxidase gene of *M. charantia.* The full-length cDNA of *McEKO* was obtained by RACE method. An 831 bp 5'-RACE product and a 955 bp 3'-RACE product were amplified based on gene specific primers designed according to the core cDNA fragment and subjected to sequence analysis. The gene was designated as *McEKO* (*M. charantia* EKO) and deposited in GenBank (GenBank accession no. GU985278).

Sequence analysis revealed that the cDNA was 1931 bp in length, having a 1563 bp open reading frame (ORF), a 151 bp 5'non-coding region and a 217 bp 3'non-coding region (Figure 1).

### Sequence analysis of putative *McEKO* protein

The putative *McEKO* protein was analyzed using the Expert Protein Analysis System Compute Pi / Mw Software and results show that the ORF encodes 520 amino acid residues (Figure 1) and the calculated molecular mass of the mature peptide was 58.8 kDa with theoretical pI of 8.40.

BioEdit 7.0 analysis software was used to analyze the amino acid of putative *McEKO* protein, of which 44.15% were neutral hydrophobic amino acids, 29.17% neutral hydrophilic amino acid, 15.74% basic amino acids and 10.75% acidic amino acids.

### Homology analysis

Based on the NCBI database BlastP program analysis, the putative amino acid sequence of *McEKO* had high identities with those from other organisms such as

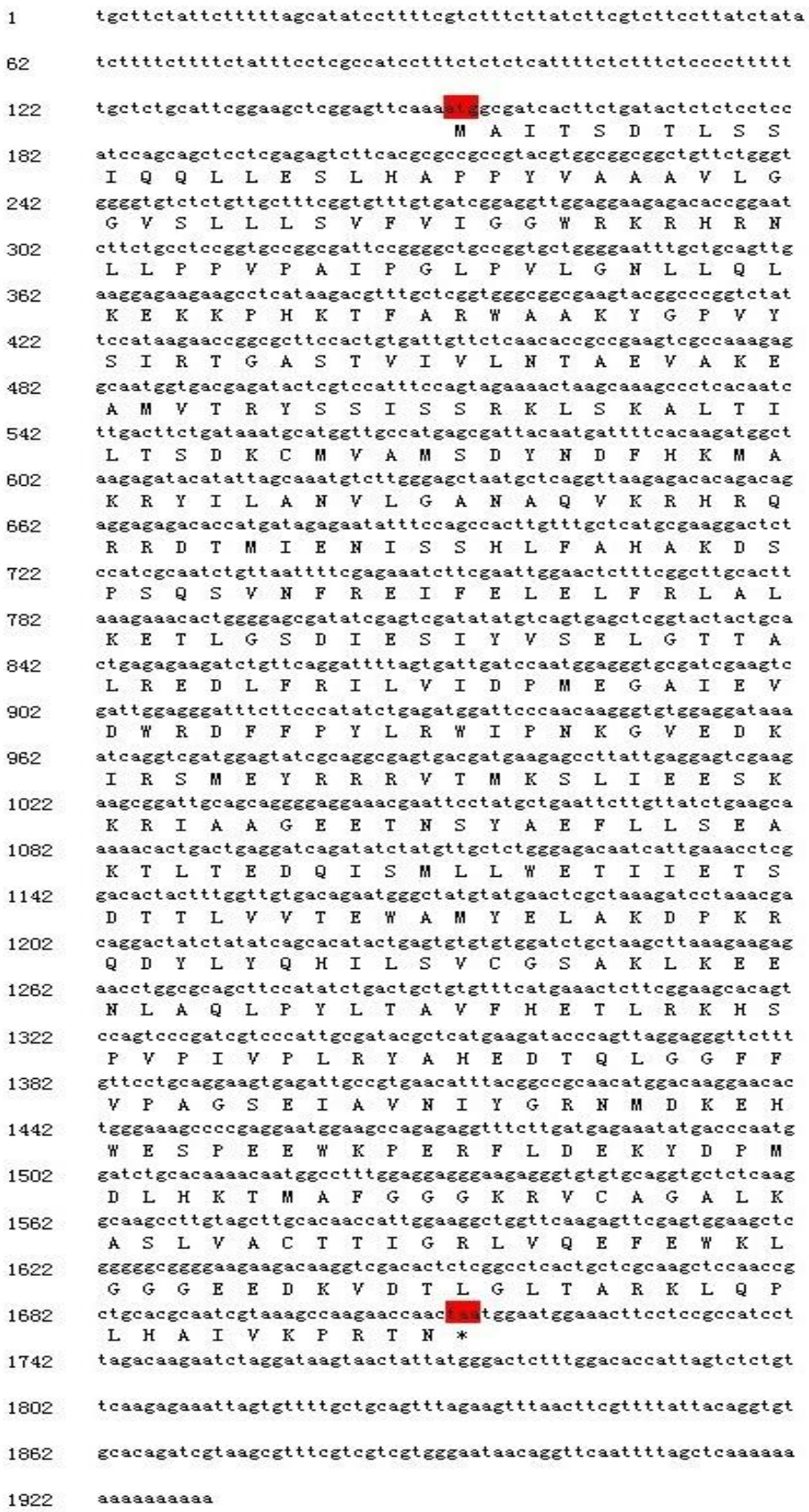


**Figure 1.** Nucleotide and deduced amino acid sequences of *McEKO* cDNA. The start codon (ATG) and the stop codon (TAA) are in shadow.

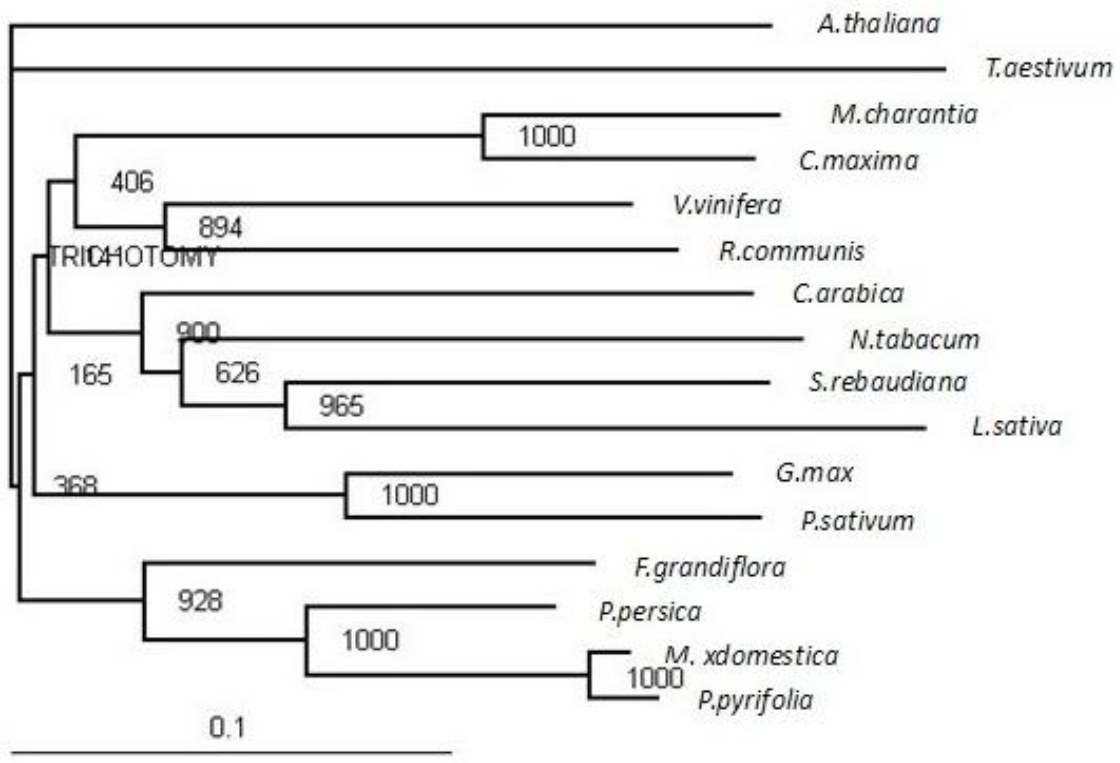


**Figure 2.** Phylogenetic tree of EKO from higher plants.

*Cucurbita maxima* (AAG41776) (80%), *Vitis vinifera* (XP002282197) (70%), *Ricinus communis* (XP002510288) (67%), Malus x domestica (AAS68017) (72%), *Glycine max* (ABC68411) (69%), *Pisum sativum* (AAP69988) (69%), *Arabidopsis thaliana* (AAC39507) (66%), *Coffea arabica* (ACQ99374) (63%), *Stevia rebaudiana* (AAY42951) (61%), *Lactuca sativa* (BAG71197) (62%), *Nicotiana tabacum* (ABC69384) (62%), *Pyrus pyrifolia* (HM003112) (71%), *Fragaria grandiflora* (AY462247) (65%), and *Prunus persica* (AF495728)(74%).

To evaluate the molecular evolutionary relationships, DNA sequences of *EKO* genes of higher plants were compared and a phylogenetic tree was constructed using Clustalx 1.8 program. As showed in Figure 2, all the EKOs are derived from a common ancestor in evolution. *McEKO* was clustered into the same subgroup with *EKO* from pumpkin (*C. maxima*), which is also a member of the Cucurbitaceae.

### Temporal and spatial transcript accumulation of *McEKO*

The transcriptional activity of *McEKO* gene was detected in seven different tissues including stem apex, root apex, leaf, male flower, female flower, neutral flower and young fruit by qRT-PCR method. Results show that the expression level in pistillate flowers was significantly higher than in staminate flowers and neutral flowers. The mRNA was especially abundant in root tip and stem tip (Table 2).

### Expression of *McEKO* in *E. coli*

The recombinant protein with a molecular weight of 58.8 kD was observed, while the negative control did not show recombinant protein bands at the corresponding point (Figure 3). The results show that the *McEKO* gene was highly expressed in *E. coli* cells.

## DISCUSSION

With the purpose of finding target gene in bitter melon related to up-regulation of early steps of the biosynthesis of GA, which is a vital group of plant hormone involved in flowering, and, hence, female flower ratio which might be manipulated by genetic engineering, *McEKO* cDNA was cloned from female flowers. In this study, the full length cDNA of *McEKO* was isolated, encoding 520 amino acid residues. Homology analysis revealed that *McEKO* had high identity with EKOs from pumpkin (*C. maxima*), which is also a member of the Cucurbitaceae. The phylogenetic tree suggested that the EKO proteins might have a similar function. Tissue-specific expression of the *McEKO* was found.

**Table 2.** Relative copy numbers of *McEKO* gene expression levels in organs of bitter melon.

| Query tissue | MC18S rRNA gene (Ct) | *McEKO* gene (Ct) | Relative copy number ($2^{-\Delta\Delta Ct}$) |
|---|---|---|---|
| Leaf | 16.37 | 27.55 | 1 |
| Root apex | 15.11 | 21.73 | 23.6 |
| Pistillate flower | 14.85 | 23.56 | 5.5 |
| Staminate flower | 16.03 | 27.08 | 1.1 |
| Neutral flower | 14.47 | 24.42 | 2.3 |
| Stem apex | 15.30 | 21.28 | 36.8 |
| Seed | 15.71 | 23.62 | 9.6 |

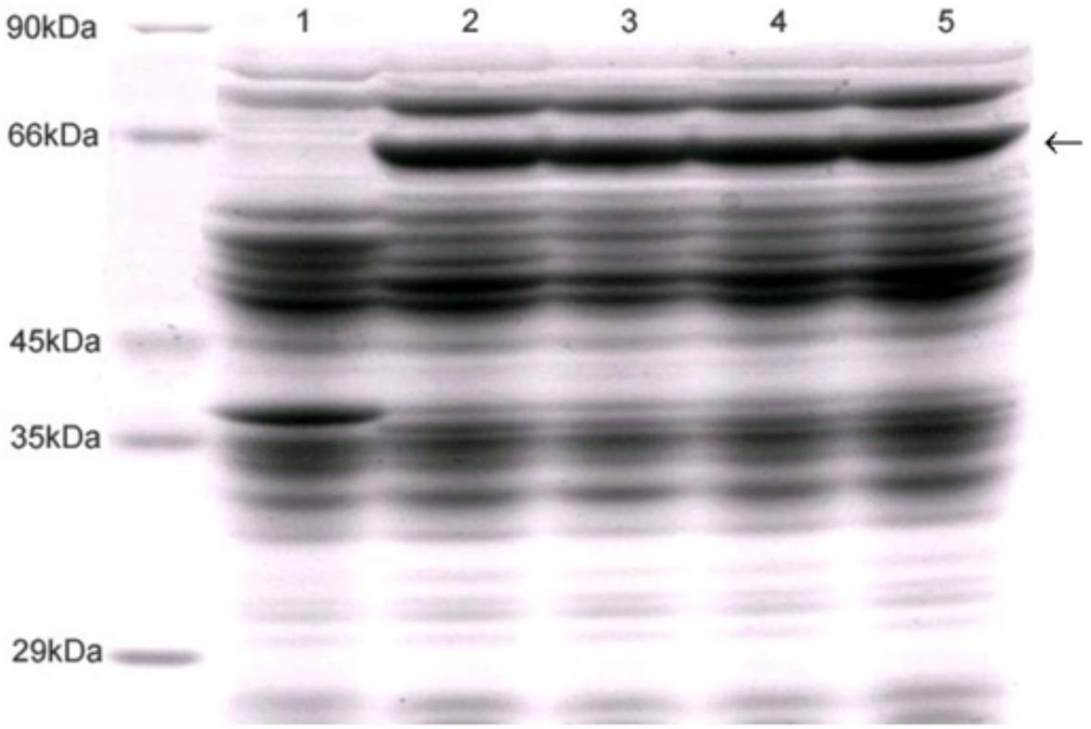


**Figure 3.** The expression of EKO induced by IPTG. 1, Negative control; 2 to 5, induced by IPTG for 1, 2, 3 and 5 h.

The highest levels of *McEKO* expression were observed in stem apex. Moreover, transcript level of *McEKO* in root apex was also high. This expression pattern suggested that the main synthesis site of GA was in the young tissues where there is a high proportion of dividing and expanding cells; the transcript was high. The expression in female flowers is higher than in male flowers and neutral flowers, suggesting that the female flowers ratio and fruit set may be increased by applied estrogen gibberellins.

We constructed the recombinant expression vector and obtained the recombinant protein in *E. coli* cells. *McEKO* was highly expressed which indicated that the enzyme could be prepared by heterogonous expression in sufficient quantities for detailed studies on their structure and function, and for the production of antibodies, which will enable us to pay attention on the protein level of *McEKO* and to investigate the relationships between the gene expression and GAs accumulation in plant tissues. Soluble analysis and purification of the recombinant protein will be performed in the next research.

Gibberellins not only promote the growth and development of plants, but also play roles in sex modification in dioecious plants. GA signaling can be used to improve crops by manipulation of the transition to reproductive growth (Mutasa-Gottgens et al., 2009). The complex pathway of GA metabolism has been investigated in depth as most of the genes involved in the metabolic pathways are identified. Over-expression of the genes coding for enzymes involved in the GA biosynthetic

pathway often results in increased levels of bioactive GAs, followed by accelerated plant development (Lange and Lange, 2006). For example, over-expression of an Arabidopsis GA 20-oxidase gene in *Arabidopsis* results in longer hypocotyls, early flowering, increased stem elongation and reduced seed dormancy (Hedden and Kamiya, 1997). EKO over-expressed *Arabidopsis* plants are partially resistant to the effects of its inhibitors (Swain et al., 2005). Over-expression of GA20-oxidase in tobacco plants shows that GA not only affects plant elongation but also seems to be positively correlated with biomass accumulation, lignin formation and the rate of photosynthesis (Sophia et al., 2004). There are no available reports revealing results concerning defect of genetic engineering of GA content up till the present moment. Whether over-expression of bitter melon *McEKO* could up-regulate biosynthesis of GA, and promote pistillate flowers proportion consequently, needs further investigation. Eukaryotic expression is underway and the results will be reported in another paper. In addition, attention will be given to the role of *McEKO* and its expression product in the process of bitter melon growth and development in further investigation.

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