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Cloning and Expression of the Ipomoelin Gene from Sweet Potato

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ABSTRACT: A full-length cDNA encoding ipomoelin protein was isolated from an enriched cDNA library, which contained wound-inducible cDNAs subtracted from that of unwounded sweet potato. Ipomoelin gene can be induced by methyl jasmonic acid, but independent of ABA. In prior studies, the expression of ipomoelin gene was found to be inhibited by salicylic acid, and related to protein kination and phosphorylation. In this study, the deduced protein sequence of ipomoelin was compared with amino acid sequences of other proteins, and it exhibited a high homology with lectin. In order to study further the function of ipomoelin, the ipomoelin gene was constructed and transformed into a bacteria expression system. The result shows that the ipomoelin protein could be over-expressed in *E.coli* one hour after IPTG induction and reached a maximum four hours after induction.

KEY WORDS: Ipomoelin, Sweet Potato, Wound-inducible, cDNA library, *E. coli* expression system.

INTRODUCTION

Plants adopt various strategies for self-protection and defense. They usually induce the expression of defense genes or elicit some chemical compounds to fight against the infection of pathogens or attack by insects. Many genes are regulated by jasmonate in response to environmental stresses, and jasmonate is involved in the octadecanoid signaling pathway in plants inducing gene expression related to the defensive mechanism (Conconi *et al.*, 1996; Farmer and Ryan, 1992; Baldwin, 1998). Jasmonate was also demonstrated to be an essential chemical for insect defense in *Arabidopsis* (McConn *et al.*, 1997). Ipomoelin was shown to be one of the wound-inducible genes in sweet potato, and was also indicated as a jasmonate-regulated gene (Imanishi *et al.*, 1997). A preliminary study indicated that some protein kinases and phosphatases might be involved in the pathway of ipomoelin expression (unpublished results). However, the function of ipomoelin in sweet potato remains unclear.

As a first step to elucidate the function of ipomoelin, attempts were made to obtain this protein through cloning by using library screening to identify the ipomoelin gene. Although this protein could be purified from sweet potato directly (Imanishi *et al.*, 1997), its purification procedure was complicated and the amount of protein obtained was not enough for functional assay. In this study, we constructed the coding region of ipomoelin gene into an expression system, and over-produced the plant protein in bacteria. This recombinant ipomoelin could be easily purified, and the quantity produced might be sufficient for its functional study.

MATERIALS AND METHODS

Sweet potato (*Ipomoea batatas* cv. Tainong 57) was wounded by crushing leaves with a hemostat, and then kept in dark for 12 hours. Total RNA of the wounded leaves was prepared with TRIZOL reagent (GIBCOBRL U.S. Cat. No. 15596) according to the procedure

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suggested by the manufacturer. Subsequently, mRNA was isolated using MicroPoly(A) Pure^{TM} kit (Ambion Cat. NO: 1918), and the cDNA synthesis mixture was incubated with 0.5 μg mRNA, 1μl SMART III oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTGG CCATTGTGGCCGGG-3'), and 1μl CDS III/3' PCR primer (5'-ATTCTAGAGGCCGAGGC GGCCGAGATG-d(T)$_{30}$N_1N-3') (N=A, G, C or T; N_1= A,G, or C) at 72℃ for 2 min. The reaction mixture was cooled on ice for 2 min before 2 μl of 5x first-strand buffer (250 mM Tris, pH 8.3, 30 mM $MgCl_2$, and 375 mM KCl), 1 μl DTT (20 mM), 1μl dNTP Mix (10 mM), and 1μl SuperScriptII reverse transcriptase were added to synthesize cDNA at 42℃ for 1 hr. The cDNA was amplified by long distance PCR (polymerase chain reaction) as suggested by the manufacturer, and the cDNA library was constructed using the SMART^{TM} cDNA library construction kit (Clontech Cat. No: K1501-1).

The cDNA library was screened by using a partial ipomoelin DNA element as a probe, which was isolated from the subtractive library prepared from the wounded leaves of sweet potato (unpublished results). The ipomoelin DNA probe was labeled with [α-^{33}P]-dCTP by PCR. Conditions used for PCR amplification involved an initial step of 5 min at 95 ℃, followed by 30 cycles of 30 sec at 94℃, 30 sec at 52℃, and 30 sec at 72℃. This probe was used to screen the cDNA library with colony hybridization, and also to check the cDNA insert by Southern blot analysis (Sambrook *et al.*, 1989).

The coding region of the ipomoelin gene from sweet potato cDNA library was constructed by the His-Patch ThioFusion^{TM} expression system (Invitrogen Cat.No:K360-01) to produce thioedoxin-ipomoelin fusion protein within TOP10 strain *E. coli.* After the bacteria containing the ipomoelin gene had been grown at 37℃ overnight, 500 μl of this culture were subcultured to 10 ml of LB medium with ampicillin, and incubated at 37℃ for another 2 hr. IPTG (isopropylthio-β-D-galactoside) was added into the medium to a final concentration of 1 mM to induce the production of ipomoelin-thioredoxin fusion protein. One ml of bacterial solution was placed on ice at 0, 1, 2, 3, 4 hr after IPTG induction. After centrifugating for 2-3 min, the cell pellet was resuspended in 500 μl cold buffer (20 mM Tris-HCl, pH 8, 2.5 mM EDTA, 5 mM imidazole). The cells were lysed by sonicator, and centrifuged to separate the soluble and insoluble parts. These two parts of the solution were analyzed respectively by 15% SDS-PAGE (sodium dodecyl sulfate polyacrylaminde gel electrophoresis).

RESULTS AND DISCUSSION

In order to check the quality of mRNA of the wounded leaves of sweet potato, the purified mRNA was analyzed on agarose gel. Although the mRNA smeared between 28S rRNA and 18S rRNA, its quality appeared to be suitable for cDNA construction (data not shown). The first cDNA strand was produced by reverse transcription using mRNA as a template, and further amplified by PCR. The products obtained from both reactions were monitored by acrylamide gel electrophoresis. These detections were needed to confirm successful synthesis of cDNA before cDNA library construction. After the cDNA was transfected into lambda phage, the number of phage plaques of this cDNA library was 2 x10^6 plaque forming units (pfu)/ml before amplification.

For a gene screening, whether the cDNA library represents all expression genes of an interesting species is an important criterion for further work. If the number of phage plaques in a cDNA library is not large enough, further screening work will have difficulty in getting the positive plaques. In this study, the titer of sweet potato cDNA library is 2 x 10^6 pfu/ml,

which is large enough to display the expression genes because the nuclear DNA content of sweet potato is 1597 Mbp/1C (Arumuganathan *et al.*, 1991). Moreover, PCR can be used to check whether the library includes the interesting gene fragment, and orient further efforts in cDNA screening.

After performing colony hybridization with the constructed cDNA library, 74 colonies showing signals were selected. However, only 27 clones were found positive by southern blotting using the partial ipomoelin gene as a probe (Fig. 1). The fragment lengths of these 27 clones were different probably because the full length of mRNA was not transcribed successfully to cDNA during reverse transcription, spatially at 5'end. Several positive clones

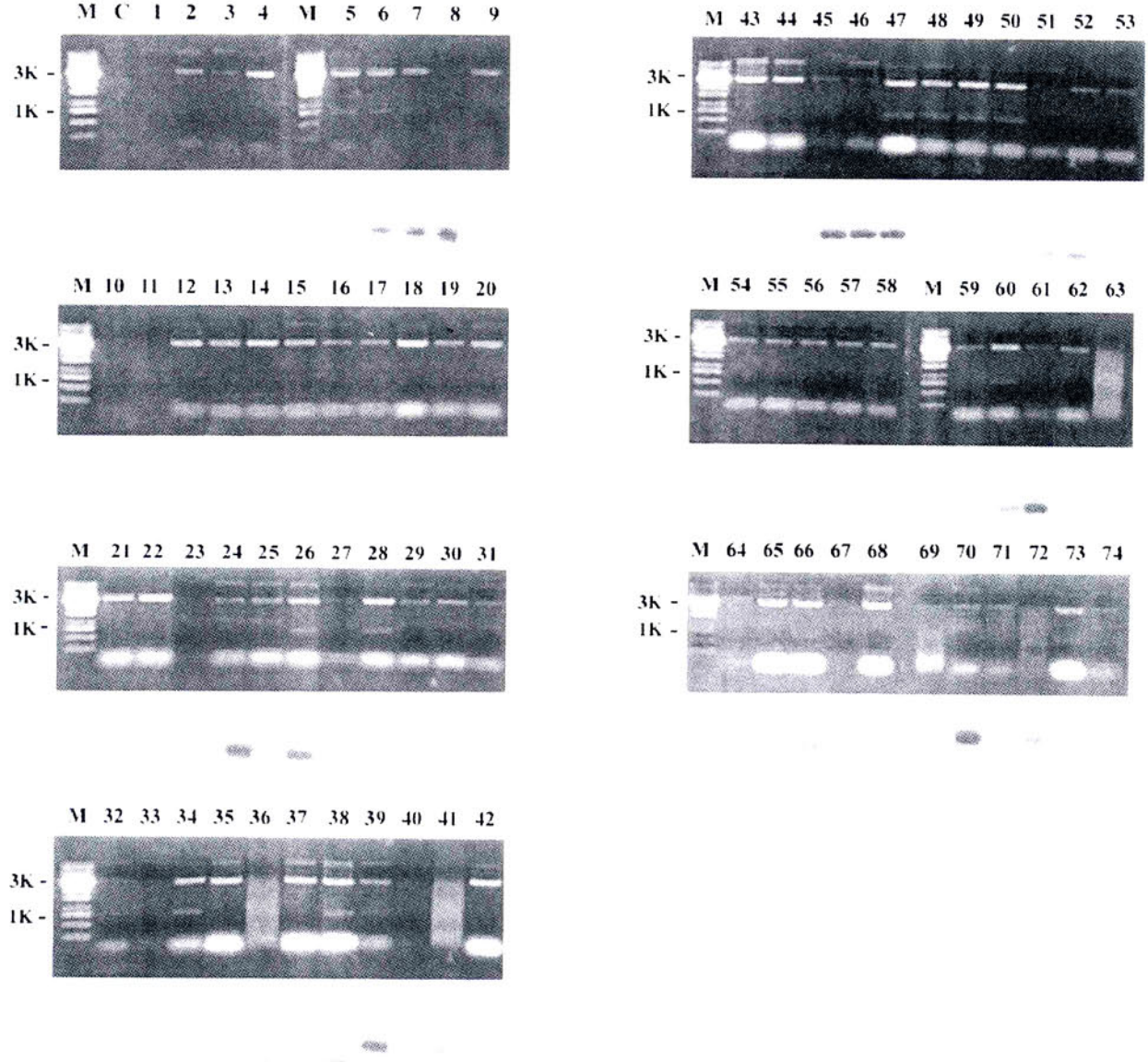

Fig. 1. Southern blot analysis of the phagemid from cDNA library. The DNA of each 74 candidates was purified from BM25.8 strain *E.coli,* which contained phagemid after *in vivo* excision. Total DNA was digested with *Eco*RI and *Xba*I, and fractionated on a 1% agarose gel stained with ethidium bromide. DNA was blotted onto a membrane and hybridized with K-^{32}P-labeled PCR product of ipomoelin gene.

were chosen for sequencing, and their sequences showed that all of them are of the same gene. Sequence from the longest insert of the positive clones was obtained for comparing with the database in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), and was found to be 100% homology with that of ipomoelin gene from sweet potato (Imanishi *et al.*, 1997).

Ipomoelin was founded in *Ipomoea batatas* Lam. Cv. Kokei No.14 (Imanishi *et al.* 1997). In this study, we found that ipomoelin was also expressed in *Ipomoea batatas* cv. Tainong 57. However, ipomoelin was not expressed in *Ipomoea batatas* cv. Tainong 67, *Ipomoea batatas* cv. Taoyuan 1, or *Ipomoea batatas* cv. Taoyuan 2 (data not shown). Since not all sweet potatoes contained it, these observations suggest that ipomoelin gene may not play a housekeeping role in sweet potato, and may have a special function in plants. The deduced protein sequence from the cDNA clone of ipomoelin exhibited a high homology with the sequences of mannose-binding lectin and jacalin (Fig. 2). Some lectins have been demonstrated to contribute to the defense mechanism of plants (Chrispeels and Raikhel, 1991). Also, plant lectin was confirmed to be involved in innate immunity (Muraille *et al.*, 1999). Therefore, ipomoelin might have similar functions as lectin, or ipomoelin itself is a lectin. To explore further the role of ipomoelin, ipomoelin was over-produced in bacteria to obtain pure protein for performing a functional assay.

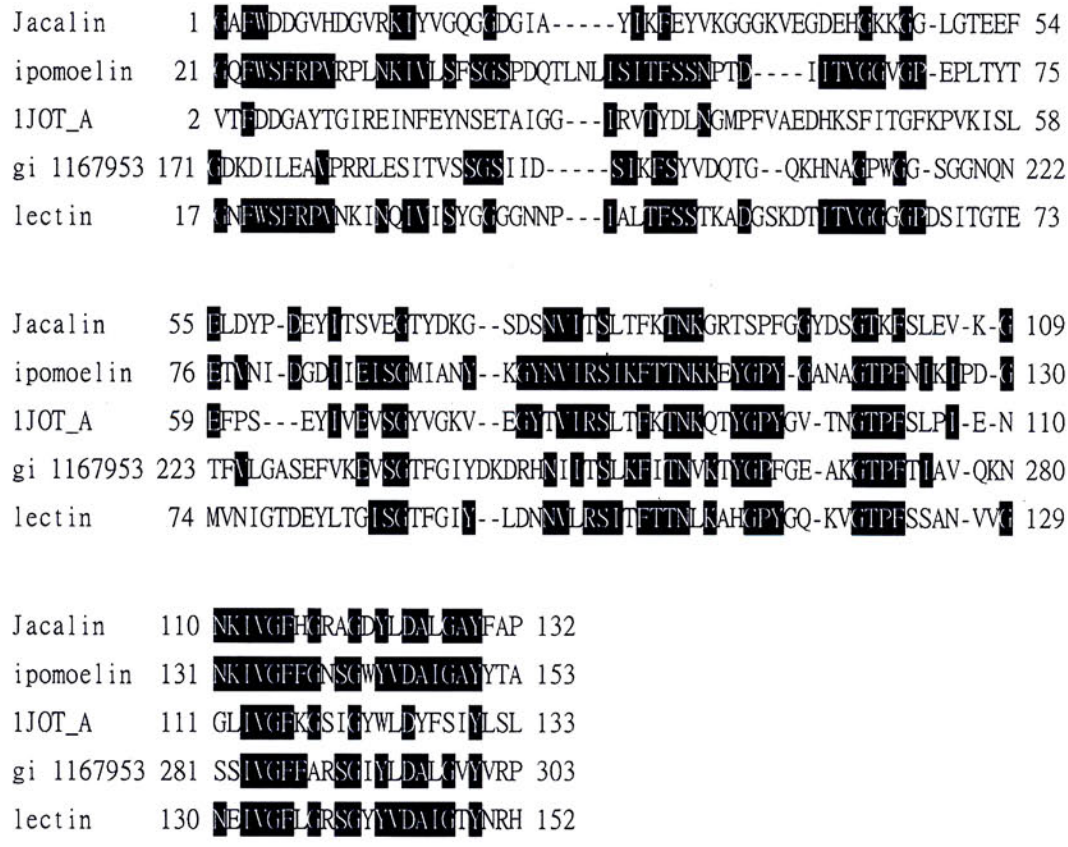

Fig. 2. Comparison of ipomoelin amino acid sequence with those of other proteins. Amino acid residues that are identical to those in ipomoelin protein are displayed as white letters in black box. Proteins for comparison include 1 JOT A in Osage orange (Accession number: 4139497), putative 32.6 kD jasmonate-induced protein in barley (Accession number: AAA87041), lectin in hedge bindweed (Accession number: AAC49564), and Jacalin in jackfruit seeds (PMID: 8673603). Result of protein BLAST in NCBI database shows that ipomoelin contains a jacalin domain from the 21 amino acid to 132 amino acid.

In order to study further the function of ipomoelin, ipomoelin was expressed as a fusion protein with thioredoxin in the His-Patch ThioFusion™ expression system. The SDS-PAGE (Fig. 3) shows that the fusion protein, thioredoxin-ipomoelin, was expressed in both soluble and insoluble parts of TOP 10 strain *E. coli* after IPTG induction, and the molecular weight of this fusion protein was about 34 kD. The maximum amount of thioredoxin-ipomoelin fusion protein was produced four hours after IPTG induction, even though the fusion protein began to accumulate one hour after induction. This thioredoxin-ipomoelin fusion protein could be digested further by enterokinase to produce ipomoelin and thioredoxin with a molecular weight of 21 kD and 13 kD, respectively (Fig. 4).

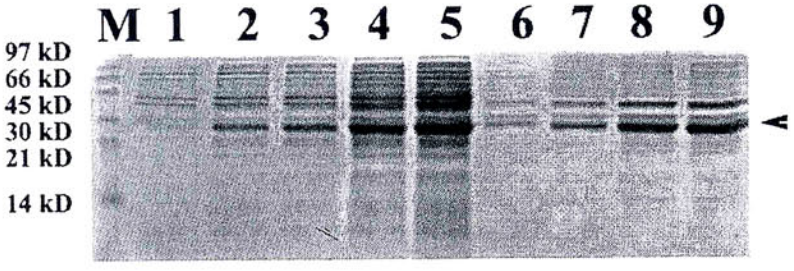

Fig. 3. Expression of ipomoelin protein in TOP10 strain *E.coli*. Total proteins were induced by 1 mM IPTG for 0 hr (lane 1), 1 hr (lanes 2 and 6), 2 hr (lanes 3 and 7), 3 hr (lanes 4 and 8), and 4 hr (lanes 5 and 9) from *E. coli*, respectively. Proteins produced were analyzed by 15% SDS-PAGE. Lanes 0 to 5 indicate the soluble protein, while lanes 6 to 9 show the insoluble protein. Arrow indicates the position of the thioredoxin-ipomoelin fusion protein, whose size is about 34 kD. Lane M is the protein markers.

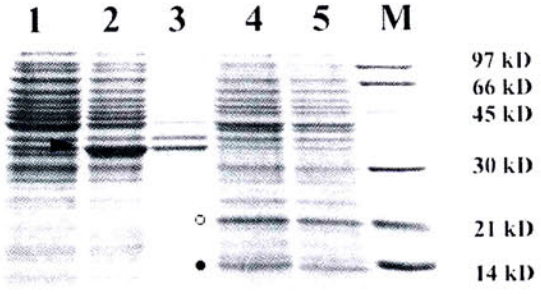

Fig. 4. Analysis of the thioredoxin-ipomoelin fusion protein digested by enterokinase. Thioredoxin- ipomoelin fusion protein from *E. coli* was digested by enterokinase enzyme at 4℃ for 16 hr. Lane 1 shows the non-induced protein extract, lane 2 indicates the soluble part of induced fusion protein extract, and lane 3 indicates the insoluble part of induced fusion protein from *E coli*. Lanes 4 and 5 show 20 μg and 10 μg of protein extract digested by enterokinase, respectively. Lane M is the protein markers. Arrow indicates the position of the thioredoxin-ipomoelin fusion protein, open circle indicates ipomoelin protein, and the closed circle indicates thioredoxin protein.

The ipomoelin protein expressed from *E. coli* (Fig. 4) was compared with that purified from sweet potato (Imanishi *et al.*, 1997). The molecular weights of ipomoelin protein from these two sources were found to be very similar. This observation indicates that ipomoelin protein produced from *E. coli* may preserve its original physiological functions. Therefore, this study further support that the over expression of ipomoelin protein from *E. coli* may be suitable for functional study.

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甘藷基因 Ipomoelin 之選殖與表現

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摘　　要

由相減式雜和反應所得到之甘藷傷害誘導表現基因已知可受甲基化茉莉酸（methyl jasmonic acid)所誘導，但卻不受離層酸(ABA)所影響，在之前的試驗中所得之 ipomoelin 基因的表現受水楊酸（salicylic acid）所抑制，並與蛋白質激酶及磷酸酶相關。在本研究中由甘藷基因庫中篩選到 ipomoelin 基因的全長，並且其蛋白質序列與其他蛋白質序列的比對發現，ipomoelin 與外源凝集素（lectin）具有高度相似性。為了更進一步研究 ipomoelin 基因的功能，將 ipomoelin 基因構築到細菌的表現系統，並由 1 mM IPTG 誘導大腸桿菌大量表現 ipomoelin 蛋白質。本文結果發現 ipomoelin 蛋白質可在 IPTG 誘導一小時後開始表現，並且在四小時的誘導後可達到最高量表現，而由此大腸桿菌系統表現出來的 ipomoelin 蛋白質的品質及產量均適宜於更進一步的功能探討研究。

關鍵詞：Ipomoelin、甘藷、傷害誘導、cDNA 基因庫、大腸桿菌表現系統。

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