RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION AND *IN VITRO* EXPRESSION OF *CAJANUS CAJAN* LECTIN GENE

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Abstract: Plants accumulate a set of defense proteins including lectins, proteinase inhibitors, amylase inhibitors etc. Lectins reversibly and non-enzymatically bind specific carbohydrates and this agglutination property makes them useful against various lepidopteran and homopteran insect pests. The isolated pigeonpea lectin (PPL) gene (~825 bp) was first cloned in pENTR-D-TOPO vector, subcloned into an expression vector (Gateway Destination vector pET300/NT-DEST) and transformed into BL21 DE3 pLysS competent cells of *E. coli* for protein expression studies. The PPL gene expression studies were carried out at different temperatures, IPTG concentrations and time intervals. The expression was maximum at 2.0 and 2.50 mM IPTG concentration at 37ºC for 5 hrs. The size of the protein was found to be around ~30 KDa. The expression was confirmed by SDS-PAGE and western blotting. Thus, transferring these defense genes under the control of tissue specific promoters will be an effective tool for sustainable insect pest management programme.

Keywords: Cloning, Expression, Insecticides, IPTG and Lectin

INTRODUCTION

Lectin genes of plant origin have an added advantage, as when transferred, they are correctly translated and processed in the plant host. Moreover, most of lectin genes are devoid of introns and therefore, both genomic as well as cDNA clones can be mobilized for developing transgenic plants. Most plants contain one or more carbohydrate binding proteins termed as lectins. They are heterogeneous group of proteins having a protective function against a wide array of organisms viz. insects, bacteria, fungi, virus etc. They reversibly and non-enzymatically bind specific carbohydrates and this agglutination property makes them useful against various lepidopteran (*Spodoptera litura, Helicoverpa armigera* etc.) and homopteran (aphids, plant hoppers etc.) insect pests but have no effect on human metabolism (Boulter *et al*., 1993). The harmful effects of lectins on biological parameters of insects include loss in weight, mortality, feeding inhibition, delays in total developmental duration, adult emergence and fecundity on the first and second generation. Realizing their importance, lectin genes have been isolated and characterized from various plant species, e.g. Snowdrop (Peumans and Van Damne, 1998), chickpea (Qureshi *et al*., 2006), mothbean (Singh *et al*., 2010) and pigeon pea (Accession number JN561784.1).

Lectins are highly specific for binding to oligosaccharides, hence if specific carbohydrate is present on the surface of tissue, it can bind to them. The ingested lectin bound to the midgut tract causes disruption of the epithelial cells including elongation of the striated border microvilli, swelling of the epithelial cells into the lumen of the gut leading to the complete closure of the lumen (Karimi *et al*., 2010).

The efficacy of the isolated pigeon pea lectin (PPL) gene needs to be tested by expressing in an expression vector and *In Vitro* insect bioassay. Then the gene coding for pigeon pea lectin will be used to develop transgenic crop plants resistant to lepidopteran and homopteran insect pests. Exploitation of these insect resistance genes for developing resistant plant varieties will provide better option for plant protection.

MATERIALS AND METHODS

Cloning PPL gene in pET300/NT-DEST vector

Isolated plasmid DNA of pigeonpea lectin gene was used for the PCR amplification using high fidelity Phusion DNA polymerase. PCR amplification was performed using gene specific primers. Forward and reverse primers were designed from the ORF region of pigeonpea lectin cDNA with the addition of CACC at the 5' end of the forward primer.

Forward primer:

5' CACCATGGCTTCTCTTCAAACCCAAA 3'

Reverse primer:

5' TGCATCTGCAGCTTGCTTAGAACTCGA 3'

The amplified PPL gene was ligated in pENTR-D-TOPO vector. The ligation reaction was prepared as per the manufacturer's instructions (Invitrogen's Gateway cloning kit, Catalog number K6300-01). The ligation mixture was kept at 22°C for 15 minutes and transformed into the supplied competent cells (one shot TOPO10). The transformed colonies obtained after overnight incubation at 37°C were picked and colony PCR was performed using Taq DNA polymerase.

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The LR recombination reaction was set up by mixing together the Entry clone, Destination vector, LR clonase enzyme mix and buffer as per the manufacturer's manual. The recombined reaction mix was transformed into one shot TOPO10 cells. The positive colonies were confirmed by colony PCR. The plasmid DNA was isolated using Macherey-Nagel plasmid isolation kit based on alkaline lysis method.

Transformation in *E. coli* BL21 DE3 pLysS cells

The *E. coli* BL21 DE3 pLysS competent cells were prepared using the TSS buffer. The isolated plasmid DNA was then used to transform the competent host cells using the 5 X KCM solution.

Protein expression and analysis

The transformed colonies were further confirmed for PPL protein expression. A nitrocellulose filter membrane was placed in close contact with the colonies on the surface of the LA plate for 10 min at RT. The filter (colony side up) was transferred to a fresh plate containing kanamycin (50µg/ml) and IPTG (1mM). The plates were incubated for 4 hrs at 37 ºC for induction of protein expression. The filters were incubated sequentially for 10 min on a sheet of 3MM Whattman filter paper soaked in SDS solution (10%), 5 min in denaturing solution, 5 min in neutralization solution (two times) and for 15 min in the 2X SSC solution. The membrane was washed and treated with blocking solution, again washed and treated with primary and secondary antibody solution. The protein expressing colonies appeared purple colored on exposure to chromogenic substrate. The transformed BL21 DE3 pLysS cells were used for induction of protein expression. The expression studies were carried out at 30ºC and 37ºC, at 3,4 and 5 hrs interval with an IPTG concentrations of 0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mM. The culture were collected, centrifuged, pellet was dissolved in sample loading buffer and loaded on SDS polyacrylamide gel (12% resolving and 5% stacking). The sample was resolved at 40 mA for 3-4 hrs, gel was separated from the glass apparatus, stained for 1 hr and then destained till the background gets clear. Western blotting was done using His-Tag specific antibodies and the colored bands developed on exposure to chromogenic substrate specific to the enzyme linked to the antibody.

RESULTS

pENTR D-TOPO Cloning of PPL and identification of positive transformants

The *E.coli* strain DH5α containing PPL gene cloned in pGEMTEasy vector was streaked on LA plates containing ampicillin (50µg/ml). The plates were incubated at 37°C. Next day the single colonies were obtained on the plates (Fig. 1a&b).

The plasmid DNA from E. coli strain DH5α containing PPL gene was isolated and purified from 10ml of overnight grown *E.coli* culture by alkaline lyses method (Birnboim and Dolly, 1979) using Macherey-Nagel plasmid isolation kit. PCR of the isolated lectin gene was performed using high fidelity phusion DNA polymerase and the gene specific primers. The forward primer had CACC at its 5'end. The amplified product of was run on 1% agarose gel at 45V for 3 hour. After the completion of electrophoresis the gel was viewed under UV light and showed a single sharp bright band at approximately 825 bp. The PCR product was used for ligation.

Ligation and Transformation

The PPL amplicon was ligated into pENTR D-TOPO vector using salt solution and incubated at 22°C for 15 minutes and transformed into TOP10 competent cells. The cells was spread on LA plates supplemented with Kanamycin and were incubated at37°C overnight. Next day, the colonies appeared on the plates. The transformed colonies were picked and streaked on fresh LA plate with kanamycin.

Colony PCR for Transformants

Colony PCR was performed to confirm the transformants. A single transformed colony was picked as a template for amplification. The amplified product of colony PCR was run on 1% agarose gel at 45 V for 3 hrs. The PCR product of 825 bp was observed on the gel (Fig. 1c). The positive colonies were used for the isolation of plasmid DNA.

Cloning of lectin gene in a Gateway Destination vector

Gateway cloning technology provides a rapid and highly efficient route to protein expression, functional analysis and cloning/subcloning of DNA segments. It is a very simple and reliable technology to clone a gene in any destination vector based on the site specific recombination reactions. The efficiency of cloning is >99%.

Isolation of Plasmid DNA from positive Transformants

The plasmid DNA containing PPL gene in pENTR D-TOPO vector was isolated using Macherey-Nagel plasmid isolation kit. The DNA was eluted in 50 µl of TE buffer. The isolated DNA was electrophoresed on 1% agarose gel to check for its quality and quantity and photographed using gel documentation system (Fig. 4.7). Quantitatively about 4 mg of plasmid was obtained per 10ml of overnight grown culture.

Transformation in Destination Vector

The LR recombination reaction mix was prepared as per manufacturers' instructions and incubated at 25ºC for 90 min. 2 µl of proteinase K solution was added to the reaction mix and incubated at 37 ºC for 10 min. The transformation was done using one shot TOP10 chemically competent *E. coli* cells and spread evenly on LA plates with kanamycin and incubated at 37ºC for 16-18 hrs. Next day the transformed colonies grew on the plate (Fig 2a).

Well separated single colonies were streaked on the fresh plate (Fig. 2b) and confirmed by colony PCR

(Fig. 2c) for the presence of the PPL gene. But for the selection of lectin protein expressing colonies and to avoid the false positives, colony blotting was done.

Transformation in *E. coli* BL21 DE3 pLysS Cells

Plasmid DNA of PPL gene cloned in Destination vector was isolated from the selected positive colonies and transformed into competent BL21 DE3 pLysS cells. The transformed cells were spread on LA plates with kanamycin (50 µg/ml), chloramphenicol (34 µg/ml) and incubated overnight at 37ºC. Colonies were observed on the plate and single colonies were streaked and confirmed for the presence of PPL gene by colony PCR.

Colony Blotting of the Transformed Colonies

The colonies were transferred onto the nitrocellulose membrane at RT. The filter (colony side up) was transferred to a fresh plate containing kanamycin (50µg/ml) and IPTG (1 mM). The plates were incubated for 4 hrs at 37 ºC for induction of protein expression. The membrane was treated with primary and secondary antibodies. Many purple colored colonies were observed on the membrane on addition of the chromogenic substrate (Fig. 3). The positive (purple colored) colonies were then selected from the master plate and streaked on a LA plate with kanamycin (50µg/ml).

Protein Expression and Analysis

Induction of Protein Expression and western blotting

The transformed BL21 colonies were inoculated in fresh LB medium and different concentrations of IPTG (0.25, 0.50, 1.0, 1.5, 2.0, 2.5 mM) were added for induction of protein expression at different time intervals (3, 4, 5 hrs.). The cells were lysed and the supernatant was used for SDS PAGE. After staining and destaining of the gel was photographed in the gel documentation system. The protein was expressed even at lower concentrations of IPTG and time period (3 hrs.), but the expression was maximum at 2.0 and 2.50 mM IPTG concentration after 5 hrs (Fig. 4),

The protein expressed was confirmed by western blotting. The protein was transferred on PVDF membrane and incubated with primary and secondary antibody solution. The membrane was incubated with chromogenic substrate until colored bands developed on the membrane. The western blot confirmed that the PPL gene was expressed in the *E. coli* expression system (Fig. 5).

DISCUSSION

To feed the ever increasing population, it is essential to increase the food production per unit area. One of the strategies is to minimize the losses due to insect pest damage. Screenings of a wide range of lectins demonstrated that several lectins have the anti-insect potential and affect the survival and development of insect pests belonging to different orders (Coelho *et al*., 2007). The anti-insect activity of plant lectins is of great potential in pest management because lectins being primary metabolic product, their genes can be good candidates to confer insect resistance to transgenic crops (Macedo and Damico, 2003). Recently transgenic rice lines resistant to sap sucking insect pest (*Nilparvata lugens*) were developed by ectopic expression of Garlic lectin (*ASAL*) in their phloem tissues (Chandrasekhar *et al*; 2014). Most widely cloned lectins belong to mannose-binding types isolated from monocots.Lectins have been cloned from seven families of angiosperms including Amaryllidaceae, Araceae, Alliaceae, Orchidaceae, Liliaceae, Iridaceae and Bromeliaceae, among which lectins from Amaryllidaceae species have been extensively studied and well documented (Van Dammeet *al*., 1998; Zhao *et al*., 2003). Many lectins are glycosylated and the presence or absence of the carbohydrate side chains can have significant effects on the functional and physical properties of the expressed proteins.

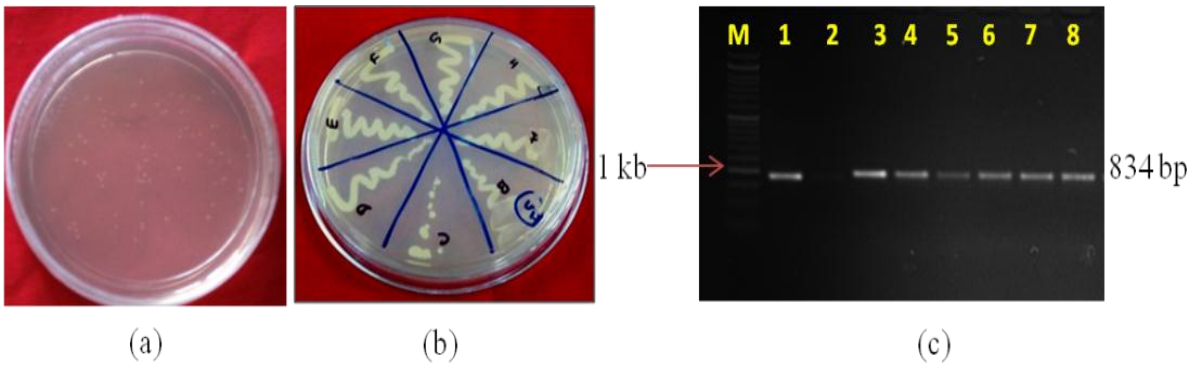

Fig 1: (a & b) Transformed colonies of DH5α strain of *E. Coli* with pENTER-D TOPO vector carrying PPL insert and (C) agarose gel showing product of Colony PCR confirming presence of insert (M- DNA ladder of 1 kb; 1-8 amplified product from +ve colony)

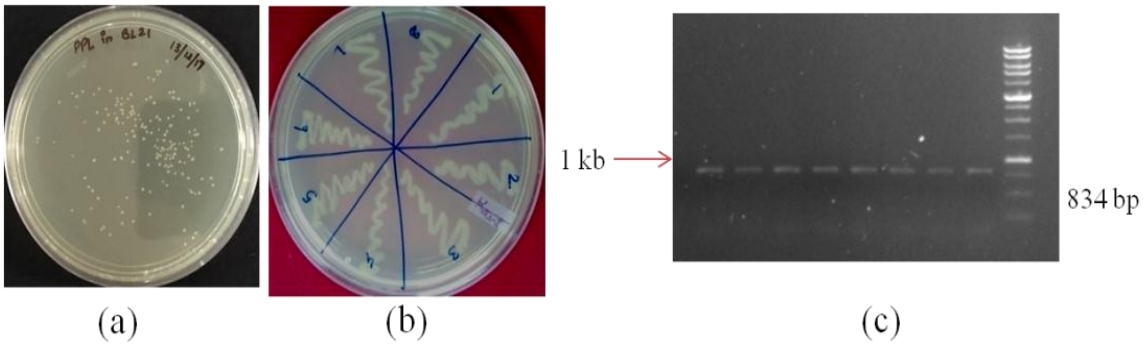

Fig 2: (a & b) Transformed colonies of DH5α strain of *E. Coli* with pET301-CT destination vector carrying PPL insert and (C) agarose gel showing product of Colony PCR confirming presence of insert (M- DNA ladder of 1 kb; 1-8 clonies)

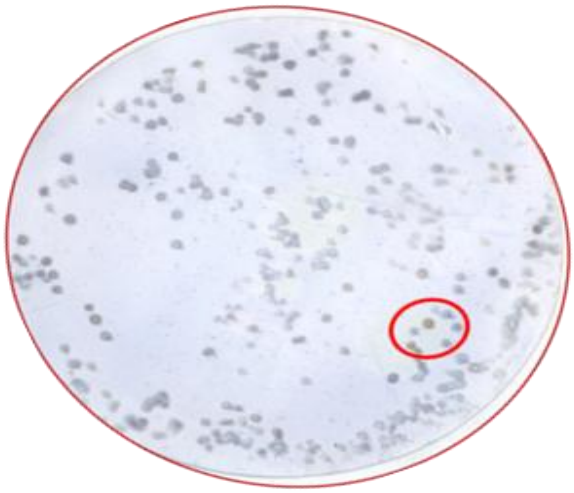

Fig 3: Colony blot of PPL proteins, where positive colonies are clearly visible (shown in red circle)

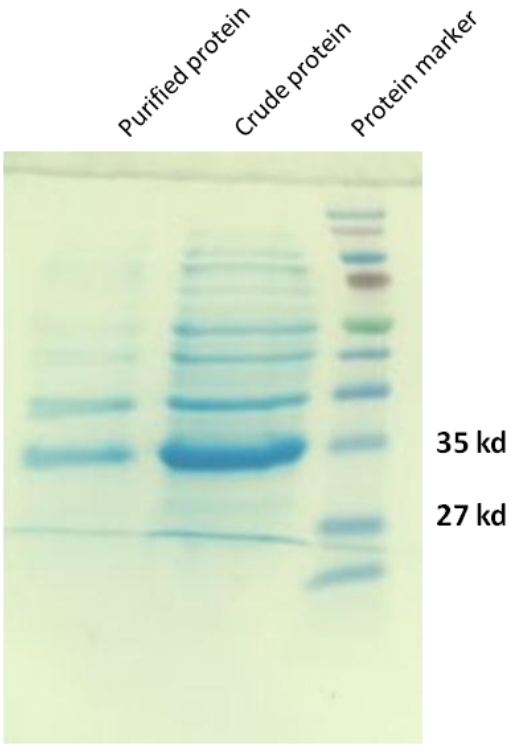

Fig 4: SDS-PAGE showing the expressed PPL protein after 4 h induction of IPTG

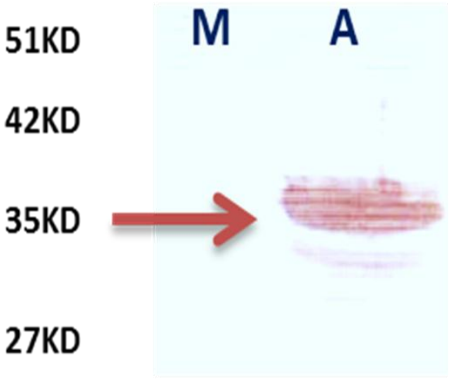

Fig 5: Western blot analysis of purified legume lectin protein on PVDF membrane using HRP-conjugated antibody with substrate DAB and H_2O_2 (Lane M- Protein ladder; Lane A-PPL)

CONCLUSION

The PPL gene (~825 bp) was cloned in an expression vector (pET300/NT-DEST) and the expression was confirmed by SDS-PAGE and western blotting. The expressed protein now will be further characterized by MALDI and insect bioassay. Then the gene can be cloned in a binary vector under tissue specific promoter and used for transformation of crop plants for developing resistance against various lepidopterans and hemipterans insect pests.

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