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Cloning of *Arabidopsis thaliana* phloem proteins *Atpp-1* and *Atpp-2*

P.B.Srividya, K.Gopinath, C.Pavan kumar, K.Pavana jyotsna.
Dept. of plant sciences, University of Hyderabad, Hyderabad, India.

Abstract— Phloem proteins that contribute to the both exogenous and endogenous signaling molecules. Role of phloem in viral particle trafficking of the plant viruses, having RNA as the genetic material. Phloem proteins having RNA binding domains. Studied from the pumpkin, *Cucurbita maxima,* having16 K.Da protein CmPP16 (*Cucurbita maxima* phloem protein 16). Exists in two types as CmPP16-1 and CmPP16-2 CMV moves long distance exclusively through phloem Potential. Involvement of a Cucumber Homolog of Phloem Protein 1 in the Long-Distance Movement CMV particle. Elucidated the role of phloem protein involved in viral transmission through Aphids. AtPP16-1(471 bp) and AtPP 16-2(444 bp) Cloning of *Arabidopsis thaliana* phloem protein (Atpp16-1 and Atpp16-2) into two versions, viz. N-His and C-His- tag. Standardization of over expression at different temperature for *Atpp16-1* and *Atpp16-2* gene.PCR amplification of N-His and C-His tag versions of Atpp16-1 and Atpp16-2. PCR amplified products of AtPP16-1 and16-2 were cloned into pTZ57R/T Cloning vector. Screening of plasmid through Blue-white selection, Plasmids isolated from white colonies Attp16-1 and Atpp16-2.Restriction digestion analysis of Positives plasmid and Conformation of pET AtPP16-1 and AtPp16-2 clones by PCR and Restriction digestion confirmation PCR confirmation of pET 28a clone of AtPP16-1 and AtPp16-2. Gene specific primers amplified the wild type clones into two versions like AtPP16-1N, 16-1C and AtPP16-2N, 16-2C.Cloning and sub cloning are successful. Phloem proteins are expressing under various temperatures, solubulizations need to be standardized. Most of the proteins are coming in inclusion bodies except one clone (16-1C).At room temperature is also phloem proteins are stable.

Index Terms—: Arabidopsis thaliana Phloem Proteins (AtPP), trafficking, amplification, restriction digestion, transformation, ligation, isolation, cloning.

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Introduction

T Viruses are very small (submicroscopic) infectious particles (virions) composed of a protein coat and a nucleic acid core. Viruses cause many important plant diseases and are responsible for huge losses in crop production and quality in all parts of the world. Infected plants may show a range of symptoms depending on the disease but often there is leaf yellowing(either of the whole leaf or in a pattern of stripes or blotches), leaf distortions(e.g. stunting of the whole plant, abnormalities in flowers or fruit formation).

Phloem is the conducting channel through which the plants transport the nutrients different organs. Plasmodesmata are the continuity between two adjacent cells there by connecting the cells for transport of molecules ans ions. Pholem and plasmodesmata thus provide a continues systemic connection even between the most distant organs of the plant. These conducting elements are often explioted by the viruses to spread systematically throughout the plant. After infection viruses normally spread from cell-to-cell through plasmodesmata until they reach the pholem.

Phloem proteins (P-proteins) are an engymatic group of proteins present in most angiosperm species. The best characterized P-proteins (PP1 and PP2) are synthesized in companion cells, transported into sieve elements via pore plasmodesmata and translocated through the plant. Characteristics such as long-distance translocation, RNA-binding activity and capacity of increasing plasmodesmata exclusion size suggest that certain phloem proteins could be involved in RNA transport within the plant, forming translocatable ribonucleoprotein complexes with endogenous or pathogenic RNAs. Here, we demonstrate that several melon phloem proteins have a wide RNA-binding activity.Translocatability and RNA-binding activity was also demonstrated for an uncharacterized protein of approximately 14 kDa**Identification of translocatable RNA-binding phloem proteins from melon, potential components of the long-distance RNA transport system**

Review Stage:

The movement machinery required for cell-to-cell spread of vRNA/DNA, in the form of nucleoprotein complexes, can be divided into at least five basic operational groups. Representative viruses for each group and their MP and ancillary proteins. This arrangement is not meant to reflect evolutionary lineages ([Koonin and Dolia, 1993], [Melcher, 2000] and [Mushegian and Koonin, 1993]), but, rather, it was developed to illustrate the increasing complexity displayed by plant viruses with respect to the identity and number of viral proteins essential for cell-to-cell movement.

Pholem Protiens

There are various protiens in the pholem that contribute to the trafficking of the both endogenous and exogenous signalling molecules. However the functions of only a few of them have been characterised. The function of most of the pholem protiens is not known yet. To date, the only con-

vincing example of most of the pholem protiens involved in signalling is the flower-promoting protien lowering locus T(FT), which was recently shown to be present in the pholem at dectable concentrations when plants were sampled at the onset of flowering (Kehr et al., 2006).

Most of the pholem protiens studied till now were extracted from the pholem sap of Pumpkin, *Cucurbita maxima*. A pholem sap in millilitre quantity extracted from pumpkin can produce sufficient data for the identification and characterization of pholem protiens. Henc it is widely chsen for the identification of pholem protiens. There are various pholem protiens that perform different functions but, among them there is a 16kDa protien called CmPP16 (Cucurbita maxima pholem protien 16), which exists in two types as CmPP 16-1 and CmPP 16-2. Both these protiens are very much similar in structure and function. These protiens have RNA binding domains that help in the trnsport of endogenous RNA and also transport their own mRNAs. It has been shown that CmPP16 is specifically expressed in the companoin cell, binds RNA, and is transported cell-to-cell by interaction with NCAPP (Non cell autonomous pathway protien 1) (Xoconostle-Cazares et al., 1999; Lee et al., 2003) But, the structure of these protiens is similar to that of the Movement protiens encoded by the viruses. Hence the viral RNA could also bind to these protiens forming RNPsand could get trafficked from cell-to-cell.

In order to identifing the homologues of these protiens in other plants especially in *arabidopsis thaliana*, and do functional charaterization *in vitro*, studies were intiated in Dr.Gopinath's research laboratory in Department of plant sciences of Hyderabad University, and identified two pholem protiens namely AtPP16-1(17kDa) and AtPP16-2(16kDa) using silico analysis. The gene sizes of AtPP16-1 and AtPP16-2 are 471bp and 444bp respectvely. Our labmates have already prepared wild type of clones and proteins. This wild type of clones are given to me to clone and produce over express altered proteins with using bacterial expression systems and standardize the conditions of solubilisation of the expressed protiens to perform in vitro binding studies using in vitro synthesized viral RNA templates.

Methods and materials

PCRAnalysis:

For amplification of AtPP16-1 and AtPP16-2 gene sequences, specific primers were designed which are used for the amplification of the respective genes.

The primer sequences were designed in such a way that the AtPP16 gene sequences will contain recognition sequences for enzymes *NcoI* and *XhoI*, This would enable us for cloning into different vectors.PCR is performed as per the protocol described by Sambrook et al.

A cocktail of 20µl for each reaction was prepared.

It contains:

Template - 0.5µl

Forward primer - 1µl (100mM)

Reverse primer - 1µl (100mM)

5mM dNTPs - 0.6µl

25mM Mgcl2 - 0.4µl

Tag polymerase - 0.4µl

10X buffer - 2µl

Milli Q - 15.9 µl

Parameters:

Initial denaturation at 94°c - 4 mins

Denaturation at 94°c - 45 sec

Annealing at 55°c - 45 sec

Extension at 72° c - 50 sec

Final extension at 72°c - 5 mins

- **Gel Extraction**:
  - Elution process was carried out by using Qiagen kit.The specific PCR bands were cut carefully from the agarose gel and dissolved in gel solubilisation buffer in 1:3 ratio and kept in water bath at 55˚c for 10min or till the gel dissolved completely.Isopropanol was added appx. 200µL and was mixed properly.The solution was transferred to the elution column and spun down at 3000rpm for 1min, thrice by putting the same solution again till the complete solution is passed through the column.The flow through was discarded and 500µl of wash buffer was added and spun down at 11,000rpm for 2min twice followed by a blank spin at 12,000rpm for 2min.The column was transferred to a fresh microfuge tube and 20µl of elution buffer was added very carefully onto the filter, incubated for 5min and was spundown at 15,000 rpm for 3min and do that as twice. Then check 1µl on agarose gel.

Ligation of PCR products into TA cloning vector:

The suitable inserts of AtPP16-1 and AtPP16-2 which are eluted from the gel are taken and ligated to TA cloning vector and plated on LB-Amp with x-gal and IPTG. After incubation white colonies were se-

lected and inoculated into LB-Amp broth medium.

Plasmid isolation:

- The plasmids were isolated as per the methodology described by Sambrook et al (1989). Alkaline lysis is the main principle involved in it.The selected colonies from the LB plate were grown overnight at 37oc in 10ml of LB medium containing ampicilin.The entire culture was centrifuged at 12,000rpm for 1min and pellet was collected.The pellet was dissolved in 400µl of P1 Solution (regeneration buffer: 50mM Tris-Hcl, pH8.8 and 10mM EDTA) with vortex in cyclomixer.450µl of P2 solution (Lysis buffer: 200mm NaoH and 1%SDS) was added to resuspended cells. The tubes were swireld gently till the solution appear clear and then allowed to stand at room temperature for 15min.Then add 500µl of P3 solution (Neutralization solution: 3M sodium acetate of pH 5.5 if RNAase is added, then it should be at 1µl/ml con..) was added. It was mixed well and incubated at 37oc for 1hr.After incubation, the solution was centrifuged at 15,000rpm for 15min at 4oc and then the pellet is removed with the help of tooth pick.Then the supernatant was collected.Isopropanol was added (600µl) up to the brim of the tubes and it was mixed well and subjected to centrifugation at 15,000 rpm for 15 minutes.The supernatant was discarded and the pellet was washed by adding 70% alcohol and the tubes were centrifuged at 15,000 rpm for 15 minutes.Supernatant was discarded and the pellet was air dried by inverting the tubes on a clean absorbent paper and letting it stand for 30 minutesAfter the pellet was air dried, it was dissolved in 30µl of 10mM Tris buffer.And 1µl is checked on agarose gel and samples are stored in freeze.

Double digestion of PTZ57R/T-TA plasmids with NcoI and XhoI;

The restriction digestion of plasmid DNA was carried out described by Sambrook et al(1989). Plasmids isolated were subjected to restriction digestion using the restriction enzymes NcoI and XhoI supplied by Fermentas in appropriate buffer and the digestion was carried at 37oc for 2hrs. After digestion the samples are analysed on 1% agarose gel.

Ligation of digested TA insert products into pET28a expressed vector:

The suitable inserts isolated from digestion of TA plasmids were ligated to pET28a expression vectors. Ligation was carried out at 4◦c for overnight using manufacturer protocol fermentas Ins TA clone kit.

pET plasmids isolation Double Digestion with *NcoI* and *XhoI:*

According to the protocols explained above are fowled here and plasmid isolation and their conformation is done by double digestion.

Transformation:

a) **Transformation into DH5α cells**: The ligated product should be introduced into a host and multiplied for further analysis. Hence, DH5α strains of E.Coli competent cells, produced by CaCl2 treatment, were selected. The method of transformation is as follows:

- 15µl of ligated product is added to 100µl of DH5α competent cells.The tube is then incubated on ice for 15min.Then heat shock treatment is given to the cells by placing the tube on a boiling water bath set at 42oc for 90sec and placed immediately on ice for 5min.The transformed bacterial cells were grown by adding 1ml of LB broth (without any antibiotic) to them and kept in shaking incubator set at 37oc and at 200 rpm for 1hr.After incubation the cells were centrifuged at 10,000 rpm for 1min and the pellet was suspended in 100µl of LB broth.The suspended culture was plated onto LB-agar plate containing appropriate antibiotic and were incubated for growth at 37oc over night.

- **Selection**: As the pET vector contains kanamycin resistance gene, we used kanamycin containing LB plates. Hence, only kanamycin resistant cells can survive forming individual colonies that can be picked up.

b**) Transformation into DE3-rosetta cells**: CaCl2 treated competent cells of DE3-Rosetta strain of E.Coli. Was used for over expression of proteins. The positive pET clones of desired genes, isolated from DH5α cells, were used for transformation. The method of transformation is as follows:

- 2µl of plasmid containing insert gene was added to 100µl of DE3-Rosetta competent cells.The mixture is then incubated on ice for 15min.Then heat shock

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treatment is given to the cells by placing them on a boiling water bath at 42◦c for 90 sec and the tube was placed immediately on ice and was left for 5-10 min.The transformed bacterial cells were grown by adding 1ml of LB-Broth to them and were kept in shaking incubator set at 37oc and at 200rpm for 1 hr.

- After incubation 100µl of culture was plated on a LB medium containing the antibiotic, kanamycine ans was incubated at 37oc for overnight growth.Single individual colonies were selected and were used for over expression.

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CONCLUSION:

The aim of the present study was to clone the *Arabidopsis thaliana* phloem proteins, AtPP16-1 and AtPP16-2 in bacterial protein expression vectors. The plasmids were transformed into DE3-Rosetta cells, which generally serve the purpose of over expressing the desired proteins.

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