Heterologous expression of $\beta$C1 of Chili leaf curl virus in Pichia pastoris

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Chili pepper is naturally susceptible to a wide range of viruses in all south Asian countries including Pakistan. Chili leaf curl virus is a monopartite begomovirus having single stranded circular betasatellite. It has one open reading frame $\beta$C1, required for pathogenicity determined, symptom induction and viral accumulation. It produces viral symptoms like mosaic, mottling, leaf distortion, vein etching, yellowing, stunting and narrowing of leaves. This study was conducted on the basis of $\beta$C1 protein, whether it was expressed in prokaryotic and yeast expression system or not because many viral proteins are lethal for the host organism. For this study, specific set of primers for $\beta$C1 were designed and amplified product was inserted into pET32a(+) bacterial and pPIC3.5K Pichia vectors for its expression. $\beta$C1 was not expressed in BL21 Escherichia coli expression system, while it was expressed in Pichia pastoris, when it was integrated into the genome through electroporation, and expressed protein was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This is the first study demonstrating the possibility of expression of $\beta$C1 protein using P. pastoris.

Key words: Monopartite begomoviruses, chili leaf curl betasatellite, heterologous expression, Pichia pastoris, betasatellite.

INTRODUCTION

Chili pepper is naturally susceptible to a wide range of viruses in all south Asian countries including Pakistan, producing various symptoms like mosaic, mottling, leaf distortion, vein etching, yellowing, stunting and narrowing of leaves (Figure I). So far, 66 viruses have been reported infecting pepper worldwide (Green and Kim, 1991). In Pakistan and some other parts of the world, ChLCB and CMV are the major viruses prevalent in chili growing areas and reducing yield up to 60, 50 and 40%, respectively (Shah and Khalid, 1999).

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Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; LB, Luria Bertani; PCR, polymerase chain reaction; IPTG, isopropyl $\beta$-D-1-thiogalactopyranoside; YPD, yeast peptone dextrose; MGM, minimal glycerol medium.

Among these, ChLCB is the most important pathogen associated with chili crop (Shah et al., 2001). Chili leaf curl virus belongs to the begomoviruses family, Geminiviridae. ChLCB is a monopatite begomoviruses having a novel type of circular single stranded satellite DNA, referred to as betasatellite. It has recently been found to be associated with some of the viruses and is required for symptom induction in plants. Betasatellite molecules are approximately 1350 nt in length (about half that of the genomes of their helper viruses) on which it depends for its replication, encapsidation, insect transmission and movement in the host plants. Numerous economically important diseases and even the earliest recorded plant viral diseases are now known to be caused by, or associated with the begomovirus/betasatellite complex (Mansoor et al., 2003). Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. Methanol is converted into formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity,
Figure 1. Chili plants showing the characteristic symptoms of virus infection like leaf curling, yellowing, vein thickening, distortion of leaves and stunting.

methanol metabolism takes place in the specialized cell organelle, called peroxisome. Alcohol oxidase has poor affinity for oxygen and *P. pastoris* compensates by generating large amount of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to derive heterologous protein expression in Pichia.

The *P. pastoris* heterologous gene expression system has been utilized to produce attractive levels of a variety of intracellular and extracellular proteins of interest. Recently, advances had improved its utility and these include: (1) methods for the construction of *P. pastoris* strains with multiple copies of AOX1-promoter-driven expression cassettes; (2) mixed-feed culture strategies for high foreign protein volumetric productivity rates; (3) methods to reduce proteolysis of some products in high cell-density culture media; (4) tested procedures for purification of secreted products; and (5) detailed information on the structures of N-linked oligosaccharides on *P. pastoris* secreted proteins (Cregg et al., 1993).

The *P. pastoris* expression system is being used successfully for the production of various recombinant heterologous proteins. Recently, developments with respect to the Pichia expression system have had an impact on not only the expression levels that can be achieved, but also the bioactivity of various heterologous proteins. Macauley et al. (2005) reviewed some of these recent developments, as well as strategies for reducing proteolytic degradation of the expressed recombinant protein at cultivation, cellular and protein levels. The problems associated with post-translational modifications performed on recombinant proteins by *P. pastoris* were discussed, including the effects on bioactivity and function of these proteins, and some engineering strategies for minimizing unwanted glycosylations.

Cregg (2007) illustrated the construction of *P. pastoris* expression strains, the general growth and manipulation of this yeast expression system in many ways similar to those of bacterial expression systems, particularly *Escherichia coli*. To aid in preventing *P. pastoris* users from falling into one or more of these traps, this introduction focuses directly on key ways on which the *P. pastoris* expression system is different. Daly and Hearn (2005) studied the different features and developments under the influences of *P. pastoris* strain selection; the choice of expression vectors and promoters; procedures for the transformation and integration of the vectors into the *P. pastoris* genome; the consequences of rare codon usage and truncated transcripts and techniques employed to achieve multi-copy integration numbers.

This research work was conducted in National Institute for Biotechnology and Genetic Engineering (NIBGE), Pakistan in molecular virology and gene silencing laboratory. In this research paper, the expression of βC1 (ChiLCV) in *P. pastoris* (yeast) expression system is described.

**MATERIALS AND METHODS**

**Cloning of βC1 of Chili leaf curl betasatellite in Bacterial expression vector**

A specific set of primers ChβC1pETF: GCGAATTCCATGCACGTTATATGAATTATGTCC having *Eco*RI and ChβC1pETR: GCAAGCTTTCACACACACATTCGTACATAC having *Hind*III was
Table 1. Polymerase chain reaction (PCR) profile.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>1Rx (µl)</th>
<th>15Rx x 5 (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>5.0</td>
<td>75.0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5.0</td>
<td>50.0</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Primer (F)</td>
<td>1.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Primer (R)</td>
<td>1.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>5.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Yeast culture</td>
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<td>50.0</td>
</tr>
<tr>
<td>ddd H₂O</td>
<td>34.6</td>
<td>519.0</td>
</tr>
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</table>

designed based on the reported sequence from Genebank nucleotide sequence database accession no. AJ316032, amplified 450 bp DNA fragment (Table 1). This fragment was cloned into T/A cloning vector pTZ57R/T (MBI Fermentas). The resultant clone was named as pSAβC1T/A. The cloned βC1 gene from pSAβC1T/A and pET32a+ vector were digested with EcoRI and HindIII restriction enzymes, precipitate with phenol : chloroform method and ligated at 16°C overnight in water bath. Ligation was transformed into 10 b competent cells through heat shock method. Transformed cells were selected on Luria Bertani (LB) medium supplemented with ampicillin (100 mg/ml). Few colonies were picked randomly from LB liquid medium supplemented with ampicillin (100 mg/ml). Cells were incubated at 37°C overnight. Next day, 1 ml of overnight culture was transferred to 2 ml broth medium supplemented with ampicillin (100 mg/ml) at 37°C. After three hours of growth, 1 mM isopropyl thiogalactopyranoside (IPTG) was added which acted as an inducer. The culture was incubated at 37°C in a shaker and allowed to grow for 8 h. Samples were taken out one by one at different time periods, pellet down the cells and frozen for overnight at -70°C. Next day, the cells were thawed and sonicated for 30 s with the interval of 30 s and placed on ice for 30 s, this process was repeated 3 - 4 times, then 1X SDS-PAGE loading dye was added. The protein samples were boiled at 100°C for 10 min, and then placed at 4°C before loaded on SDS-PAGE gel analysis.

Cloning of βC1 of ChLCB in P. pastoris vector

The resultant clone pSAβC1PET32a (+) and pPIC3.5K were digested with EcoRI and NotI restriction enzymes. The digestion was run on 1% agarose gel and restricted fragment of expected size was eluted from the gel by DNA extraction kit (MBI Fermentas). Elution was confirmed by running 2 µl of gel extracted DNA on 1% agarose gel and purified fragment was ligated into P. pastoris expression vector at EcoRI and NotI. Ligation was transformed into 10 b heat shocked competent cells and colonies were selected on LB medium supplemented with ampicillin (100 mg/ml). The resultant recombinant vector was named pSAβC1PIC3.5K.

Transformation and screening of βC1 of ChLCB in P. pastoris GS115 strain

Construct pSAβC1PIC3.5K plasmid was isolated from E. coli strain by miniprep method, linearized with NotI restriction enzyme for integration in the genomic DNA of P. pastoris. Electroporation competent cells of P. pastoris GS115 strain were prepared by electrochemical method. The electroporation apparatus was adjusted to 2.0 kV. Electroporation cuvettes with a gap of 2 mm were used in this experiment. The GS115 electro competent cells (80 µl as removed from the -70°C freezer and thawed on ice) were transferred and pSAβC1PIC3.5K was linearized in the cuvettes, electric shock was given at 2.0 kV. Then immediately, 1 ml 1 M of sorbitol was added and the cuvettes were placed on shaker at 30°C for 2 h. After 2 h, the medium was centrifuged at 13200 rpm for 1 min to collect pellet. Pellet was dissolved in 100 µl 1 M sorbitol and spread on the yeast peptone dextrose (YPD) agar plates containing antibiotic geneticin up to final concentration of 0.25 mg/mL and these plates were transferred to incubator at 30°C until colonies appeared. Selection of transformants was carried out at different concentrations of geneticin antibiotic in YPD agar medium plates. These concentrations were 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mg/mL, respectively.

Optimization of P. pastoris growth for SDS-PAGE analysis

P. pastoris transformants having pSAβC1PIC3.5K were picked from 0.75 mg/mL geneticin YPD agar media plates along with non-transformant GS115 as a control and cultured into YPD broth media without antibiotic at 30°C (OD600 = 1.0). Cells were harvested by centrifugation at 3000 rpm for 5 min at room temperature. Supernatant was discarded and cell pellet was resuspended into 25 ml minimal glycerol medium (MGM) and 0.02% 10 X dextrose in a 100 ml flask. Cultures were placed at 28 - 30°C in a shaking incubator (150 - 200 rpm) until growth reached log phase. Once the cells are in log phase, they can be induced for βC1expression. 1 ml culture was taken before each induction of 100% methanol to a final concentration of 30 µl in 25 ml MG medium. Induced culture was collected at different time intervals (24, 48, 72, 96 and 120 h), respectively. 1 ml of expression culture was transferred into 1.5 ml microcentrifuge tubes. The apparatus were adjusted to 2.0 kV. Electroporation cuvettes with a gap of 2 mm were used in this experiment. The GS115 electro competent cells (80 µl as removed from the -70°C freezer and thawed on ice) were transferred and pSAβC1PIC3.5K was linearized in the cuvettes, electric shock was given at 2.0 kV. Electroporation cuvettes with a gap of 2 mm were used in this experiment. Then immediately, 1 ml 1 M of sorbitol was added and the cuvettes were placed on shaker at 30°C for 2 h. After 2 h, the medium was centrifuged at 13200 rpm for 1 min to collect pellet. Pellet was dissolved in 100 µl 1 M sorbitol and spread on the yeast peptone dextrose (YPD) agar plates containing antibiotic geneticin up to final concentration of 0.25 mg/mL and these plates were transferred to incubator at 30°C until colonies appeared. Selection of transformants was carried out at different concentrations of geneticin antibiotic in YPD agar medium plates. These concentrations were 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mg/mL, respectively.

Preparation of βC1 of ChLCB protein sample in P. pastoris GS115 strain

The sample was prepared for SDS-PAGE, as cell pellets were thawed quickly and placed on ice. Pellet was dissolved in 1 ml distilled water and 100 µl breaking buffer. An equal volume of acid washed glass beads (size 0.5 mm) was added. It was vortex for 30 s, incubated on ice for 30 s (repeated for several times) then
RESULTS

Expression of βC1 through prokaryotic system

Cloning of βC1 gene into pET32a (+) vector (bacterial expression system)

The PCR amplified 450 bp fragment using specific primers (Figure 2A) was cloned into T/A cloning vector pTZ57R/T. The resultant recombinant clone pSAβC1T/A, was confirmed through digestion (B). After digestion with EcoRI and HindIII, the βC1 gene was cloned in the pET expression vector and verified with restriction enzymes (C). Finally pSAβC1pET32a(+) recombinant clone transformed into E. coli strain BL21 for bacterial expression. BL21 transformants having the target gene from pSAβC1pET32a(+) was confirmed through PCR analysis by using set of specific primers (D).

SDS-PAGE analysis for βC1 protein induced by IPTG inducer

After transformation and confirmation of pSAβC1pET32a (+) and pET32a(+) in E. coli strain BL21, the samples were processed and analyzed on SDS-PAGE. The desired band could not be detected in the induced transformants having pSAβC1pET32a (+) with increase...
in time after 1 mM IPTG induction of 30, 60, 90 and 120 min, respectively. On the other hand, the other bands regarding to BL21 proteins were visible. We increased the level of IPTG and time of induction up to 7 h to expression βC1 in bacteria, but the experiment failed to detect protein on SDS PAGE analysis. However, βC1 was easily cloned into pDONR/Zeo gateway vector through gateway cloning, but it also failed (data not shown).

Expression of βC1 through P. pastoris system

Isolation of βC1 gene and construction of pSAβC1PIC3.5K recombinant vector

Both construct pSAβC1pET32a(+) and vector pPIC3.5k was digested with EcoRI and NcoI. Upon digestion of pSAβC1pET32(+), it produced 450 bp fragment from the vector backbone of pET32a(+) vector, whereas, pPIC3.5k produced a linear fragment of approximately 9000 bp. Restriction analysis indicated that EcoRI and NcoI are unique multiple cloning restriction sites of pPIC3.5k, whereas, in pSAβC1PIC3.5K it is located on upstream and downstream of gene. The elution of the target fragments of approximately 450 and 9000 bp, respectively, was carried out through DNA extraction kit (Fermentas) and eluted bands were analyzed through running on 1% agarose gel electrophoresis. Eluted products were ligated at 16°C. βC1 gene from pSAβC1pET32 was cloned at the same sites of EcoRI and NcoI in pPIC3.5K. The resultant vector was named pSAβC1PIC3.5K.

Confirmation of clone pSAβC1PIC3.5K in E. coli TOP10F’ strain

Ligation product was transformed into E. coli TOP10F’ strain by heat shock method. The recombinant clone pSAβC1PIC3.5K was confirmed through digestion analysis.

Upon digestion with EcoRI and NcoI, recombinant clone pSAβC1pPIC3.5K produced 450 bp fragment along with vector backbone of pPIC3.5K of 9000 bp, mobilized into E.coli TOP10F’ strain for its mass production before transforming in P. pastoris GS115 strain.

Expression of βC1 gene in P. pastoris (yeast expression system) GS115 strain: Transformation and selection of recombinant Pichia clones carrying foreign gene through PCR

pSAβC1pPIC3.5K plasmid was isolated from TOP10F’ E. coli strain and linearized with NcoI restriction enzyme. The linearized fragment was treated with phenol: chloroform for purification and transformed into P. pastoris strain GS115 through electroporation. Concentration of geneticin was optimized for the selection of transformants from 30, 50, 70, 90, 110 and 130µg/ml. Suitable concentration of geneticin antibiotic for selection of transformants was found to be 0.25 mg/ml. The transformants were grown on YPD agar media plates containing 0.75 mg/ml geneticin antibiotic.

Confirmation of the transformants in P. pastoris GS115 strain

The integration of linearized fragment pSAβC1pPIC3.5K having βC1 gene in the genome of P. pastoris was confirmed through PCR amplification by using βC1-specific primers (Figure 3). The amplified 450 bp fragment from the transformants of P. pastoris having pSAβC1PIC3.5K was confirmed through 1% agarose gel electrophoresis.

Sequence analysis through Fasteris SA, Plan-les-Ouates, Switzerland

After confirmation of the exact size of the βC1 in pDONR/
Zebras were kept in small groups to prevent them from feeling threatened. The expression was induced by the addition of 100% methanol in minimal glucose broth media without geneticin. The strains transformed by parent plasmid pPIC3.5K were taken as control. The samples were collected at different times of induction (after 24, 48, 72 and 96 h), respectively. These samples were processed and analyzed on SDS-PAGE. The intensity of desirable protein approximately 26 kDa was increased with increase in time after 100% methanol up to a final concentration of 0.5% induction. The band of the same molecular mass could not be detected in the induced recombinant strain transformed pPIC3.5K vector and the results showed that the recombinant protein was successfully expressed (Figure 5). The protein bands were clearly visible only in the case of the sample that was incubated for 72 h and no clear bands were observed after 24, 48 and 96 h of incubation. Studies conducted further to scale-up the expression level with increased induction period and methanol concentration revealed 72 h of post-induction incubation period with 1.5% methanol concentration for large amount of βC1 protein expression. However, the level of expression could not be further boosted above this scale with either increased methanol concentration or increased duration of incubation.

**DISCUSSION**

**Bacterial expression of βC1**

Betasatellite gene βC1 was successfully cloned into bacterial expression vector pET32a (+) and transformed into BL21 *E. coli* strain for expression, but βC1 was not expressed in prokaryotic system after several trials experiments. Similar results were obtained when we cloned βC1 through gateway cloning vector. We observed that when we cloned βC1 into pDONR/Zeo vector after confirmation through PCR and restriction analysis, it produced same fragment of βC1, but it showed nucleotide mutations through sequence analysis after several trials of cloning. Figure 4A describes the alignment of different clones of gateway vectors having βC1 through clustal W (1.8) aligner program at http://www.justbio.com/tools.php and also aligned through above clustal W (1.8) program. The proteins alignment demonstrates the changes in the order of amino acids as indicated in (Figure IV. B).

**Confirmation of βC1 gene expression**

The expression of targeted gene in *P. pastoris* GS115 strain transformants having recombinant vector pSAβC1p PIC3.5K was confirmed from four selective GS115 cultures. The expression was induced by the addition of 100% methanol in minimal glycerol broth media without geneticin. The strains transformed by parent plasmid pPIC3.5K were taken as control. The samples were collected at different times of induction (after 24, 48, 72 and 96 h), respectively. These samples were processed and analyzed on SDS-PAGE. The intensity of desirable protein approximately 26 kDa was increased with increase in time after 100% methanol up to a final concentration of 0.5% induction. The band of the same molecular mass could not be detected in the induced recombinant strain transformed pPIC3.5K vector and the results showed that the recombinant protein was successfully expressed (Figure 5). The protein bands were clearly visible only in the case of the sample that was incubated for 72 h and no clear bands were observed after 24, 48 and 96 h of incubation. Studies conducted further to scale-up the expression level with increased induction period and methanol concentration revealed 72 h of post-induction incubation period with 1.5% methanol concentration for large amount of βC1 protein expression. However, the level of expression could not be further boosted above this scale with either increased methanol concentration or increased duration of incubation.

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**P. pastoris** expression of βC1

The *P. pastoris* system used in the present study is able to utilize methanol as its sole carbon source and has been widely used as a host for the expression of heterologous proteins. In this study, the βC1 gene of ChLCB2 was inserted towards the downstream of AOX1 promoter of the expression vector pPIC3.5K and the chimeric construct was integrated into the host genome through homologous recombination. The recombinant yeast can also perform many eukaryotic post-translational modifications in the target protein, such as glycosylation, disulfide bond formation and proteolytic processing. Previous studies demonstrated that *P. pastoris* was capable of expressing glycoproteins processed in a manner similar to virus infected insect cells (Trimble et al., 1991). As the target gene is integrated within the genome, it is difficult to lose the target gene when the recombinant yeast is cultured. Therefore, *P. pastoris* has been used successfully to express a wide range of heterologous proteins (Andleeb et al., 2008; Romanos, 1995; Hagenson et al., 1989; Sreekrishna et al., 1997). Heterologous expression in *P. pastoris* can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretary pathway. Although all four positive colonies were selected for induction, only two colonies showed expression of the target protein and the remaining two did not show detectable levels of the target protein in the gel for which reasons are not known.

The actual size of the gene 450 bp has been confirmed through PCR analysis (Figure 3) and the expected size of protein should be 16.5 kDa but SDS-PAGE analysis showed approximately 26 KDa molecular weight of a specific protein, which is expressed in *P. pastoris*, whereas, the protein sample transferred from *Pichia* transformed
Figure 4: Alignment of nucleotide and amino acid sequence analysis of βC1 in gateway vectors. (A) Indicates the nucleotide sequence homology of recombinant clones of gateway vector having βC1 gene through Clustal W (1.8) program with reported βC1 of NIB16. With solid deep red shade exactly matched with NIB16 and solid dark green color indicated mismatched with NIB16. (B) Shows the amino acid homology of recombinant clones of gateway vector having βC1 gene through Clustal W (1.8) program with reported βC1. Colorless amino acid indicates the differences between the protein sequences.

with pPIC3.5K negative control did not develop any signal on the SDS membrane (Figure 5). The protein expression in P. pastoris was secreted and not intracellular. There may have been glycosylation which would make the protein larger and possibly produce multiple bands through multiple integration of pPIC3.5k recombinant vector with genomic DNA of Pichia. In order to increase the expression level, the expression conditions were optimized. The protein bands were clearly visible only in case of the samples that were incubated for 72 h and no clear bands were observed before or after 72 h of incubation. This could be due to the critical incubation
period, that is, 72 h required for biomass accumulation. Very faint but multiple bands were observed following 72 h and no bands were visible after 120 h of incubation indicating that host-specific proteases may be acting on the protein following prolonged incubation. The observed low level of βC1 expression may be partly attributed to the low copy number of the genes integrated within the yeast genome and partly to the nature of the protein. The most important parameter for efficient expression in Pichia was found to be adequate aeration during methanol induction and hence the culture volume within the flask was kept as low as 20% of the total flask volume. It was also necessary to maintain the incubation temperature at 28°C with rotation of 250 – 300 rpm, above this temperature, yeast does not survive.

Conclusion

In conclusion, the pSAβC1PIC3.5K recombinant protein has been expressed for the first time in both bacterial and yeast expression systems in our laboratory of Molecular Virology/Gene silencing (NIBGE, Pakistan). Our studies indicate that P. pastoris can be an excellent expression system for recombinant proteins. The P. pastoris expression system offers economy, ease of manipulation, the ability to perform complex post-translational modifications and high expression levels. Its use as an expression system will become increasingly more efficient and user-friendly. On the other hand it was also observed through sequence analysis that βC1 gene of ChLCB had mutations in nucleotide sequence when expressed in the bacterial system and could not be produce required protein and in modified form. It was suggested that may be it is due to its toxic effect in the system. The pSAβC1PIC3.5K recombinant vector constructed in the present study is suitable for secretory expression of the target protein but the expression of target protein was low and could not be purified. So, in future, we can modify the system for overproduction of pSAβC1PIC3.5K, to purify and use it in binding studies.

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