Expression of tomato yellow leaf curl virus coat protein using baculovirus expression system and evaluation of its utility as a viral antigen

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Abstract DNA encoding the coat protein (CP) of an Egyptian isolate of tomato yellow leaf curl virus (TYLCV) was inserted into the genome of Autographa californica nucleopolyhedrovirus (AcNPV) under the control of polyhedrin promoter. The generated recombinant baculovirus construct harboring the coat protein gene was characterized using PCR analysis. The recombinant coat protein expressed in infected insect cells was used as a coating antigen in an indirect Enzyme-linked immunosorbent assay (ELISA) and dot blot to test its utility for the detection of antibody generated against TYLCV virus particles. The results of ELISA and dot blot showed that the TYLCV-antibodies reacted positively with extracts of infected cells using the recombinant virus as a coating antigen with strong signals as well as the TYLCV infected tomato and beat plant extracts as positive samples. Scanning electron microscope examination showed that the expressed TYLCV coat protein was self-assembled into virus-like particles (VLPs) similar in size and morphology to TYLCV virus particles. These results concluded that, the expressed coat protein of TYLCV using baculovirus vector system is a reliable candidate for

generation of anti-CP antibody for inexpensive detection of TYLCV-infected plants using indirect CP-ELISA or dot blot with high specificity.

Keywords Tomato yellow leaf curl virus · Baculovirus expression system · Coat protein · ELISA test

Introduction

Tomato is one of the most important cultivated economical vegetable all over the world. The most destructive disease of tomato is TYLCV which is transmitted by whitefly *Bemisia tabaci* and exclusively infects dicotyledonous plants (Al-Amri 2013; Czosnek and Laterrot 1997; Shafiq et al. 2010; Diana et al. 2013; Kil et al. 2016). It can be found in tropical and subtropical regions causing severe economic losses. TYLCV belongs to the family Geminiviridae genus *Begomovirus*. This family is characterized by having single-stranded circular DNA genome of about 2.8 kb encapsidated in a twinned icosahedral virion (Moriones and Navas-Castillo 2000). Symptoms of geminivirus infection vary depending on virus type and strain, plant cultivar, plant age, time of infection and environmental conditions. TYLCV-infected tomato plants show severe symptoms on plants such as leaf rolling, leaf curling and yellowing, reduction in leaf size, stunting of the infected plant and flower abscission which dramatically cause production loss in tomato cultivation (Polston and Anderson 1997). In addition to tomato as a main host, there are other crops that have been considered as TYLCV hosts such as common bean (*Phaseolus vulgaris*), cucurbit (*Cucumis* species), pepper (*Capsicum* species) and eustoma (*Eustoma grandiflora*) (Moriones and Navas-Castillo 2000; Kil et al. 2016).

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Virus particles are normally used as an antigen for polyclonal and monoclonal antibody production to be used subsequently for seriological diagnosis of TYLCV virus-infected plants. However, purification of virus particles is usually time-consuming and the end results may vary concerning the purity and concentration of the final product (Ling et al. 2007). To overcome these difficulties, different methods have been applied including the use of recombinant DNA technology to express the virus coat protein (CP) in prokaryotic or eukaryotic cell systems. Hence, the purified protein can be successfully used for antisera production (Alves-Junior et al. 2008). The end product yield and biological activity of the obtained recombinant protein normally depends on different factors such as protein folding, stability and solubility (Canto et al. 1997). Therefore, developing an efficient system for early detection of TYLCV in infected plants will dramatically assist in controlling viral spreading in tomato plants which significantly reduce crop losses. Baculovirus expression vector system (BEVS) is one of the most efficient protein expression methods that successfully used for different foreign protein expression. In this study, we used baculovirus vector system for TYLCV-coat protein expression in insect cell culture to examine its specificity as a viral antigen against TYLCV-antibodies.

Materials and methods

PCR amplification of TYLCV-CP gene

Genomic DNA of TYLCV, isolated from infected tomato plants, was used as a template for PCR amplification of coat protein gene (CP). Two specific primers, denoted TYLCV-Cp-F 5'-CGGAATTCACTATGTCGAAGCGACCAGG-3' and TYLCV-Cp-R 5'-CGGGATCCTTAATTTGATATTGAATC-3' were designed upon the published sequences under the accession number AY594174.1. *EcoRI* and *BamHI* restriction sites (underlined) were added to the 5'-end for the forward and reverse primer, respectively. The PCR reaction was performed in a 25- μ l mixture containing specific forward and reverse primers (10 pmol each), 5 $\times$ PCR reaction buffer, 1.5 mM $MgCl_2$, 2.5 mM dNTPs mixture, 1.5 units GoFlexi Taq DNA Polymerase (Promega) and 5 μ l of viral genomic DNA. Typical PCR program was used in an initial cycle of 94 °C for 5 min and 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C followed by final extension at 72 °C for 10 min. The obtained CP gene PCR fragment was gel-purified using the Qiaquick gel extraction kit (Qiagen), cloned into pGEM-T vector (promega) and analyzed by nucleotide sequences. The CP gene fragment was released from pGEM-T-CP construct to be subcloned

into pFastBac-Dual vector under Polyhedrin promoter using *EcoRI* and *BamHI* restriction sites.

Generation of the recombinant bacmid

In order to generate the recombinant bacmid harboring the TYLCV-CP gene, the recombinant plasmid pFBD-polh-TYLCV-CP was transformed into DH10Bac competent *E. coli* using the manufacturer's instruction (Bac-to-Bac manual, Life technologies). One nanogram of the pFBD-polh-TYLCV-CP DNA was added to 100 μ l of the pre-chilled competent DH10Bac cells. The mixture was incubated in ice for 30 min and heat-shocked for 50 s at 42 °C. Immediately after cells were heat-shocked, the mixture was chilled on ice for 2 min. About 900 μ l of LB medium was added to the mixture and incubated at 37 °C with shaking for 4 h to facilitate transposition of the recombinant cassette into the bacmid mini-*attTn7* site. Five serial dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5}) of the obtained culture were performed and spread on LB-Agar plates containing (50 μ g Kanamycin, 7 μ g Gentamycin and 10 μ g Tetracycline). To facilitate blue/white colonies' screening, X-Gal (100 mg/ml) and IPTG (40 μ g/ml) were added to each plate before spreading of the culture. All plates were incubated for 48 h at 37 °C. To verify successful transposition, nine clear white colonies were PCR analyzed using one pair of pUC/M13 primers designated M13 Forward 5'-(GTTTTCCTCAGTCACGAC)-3' flanking the right border of the mini-*attTn7* transposition site, and TYLCV-Cp-R, the CP gene-specific reverse primer. The recombinant bacmid DNA was isolated from 2 ml of overnight culture of single positive white colony following the manufacturer's instruction (Life technologies).

Transfection and expression of TYLCV-CP gene in Sf9 cells

The verified recombinant bacmid was transfected into Sf9 cells using the method described by O'Reilly et al. (1992). Briefly, purified recombinant bacmid DNA (500 ng) and Cellfectin transfection reagent (Life technologies) were mixed in a total volume of 210 μ l of Excell-420 serum-free medium and kept for 30 min at room temperature. The mixture was added drop wise to Sf9 cells (5×10^9 Cells/plate) previously grown in 35-mm tissue culture 6-well plate. The cells were incubated at 27 °C for 5 h; then the medium containing Cellfectin/bacmid DNA mixture was removed and replaced with Excell-420 medium containing 3% Fetal bovine serum (FBS) and the appropriate antibiotics. Cells were incubated at 27 °C for 72 h in a humidified incubator until appearance of signs of viral infection. The supernatant containing budded virus (BVs) of the recombinant bacmid was separated from Sf9 cells (P1 viral stock). The

supernatant was used to prepare P2 and P3 viral stocks via serial infection of Sf9 cells for 2 weeks to increase virus titer. The recombinant virus stock P3 was used to infect Sf9 cells with multiplicity of infection (MOI) of 10 for 72 h and the Sf9 cells were harvested and applied for total protein extraction.

ELISA and dot blot

Indirect ELISA and dot blot analysis were used to evaluate the specificity of the expressed TYLCV-CP in immunoreaction against TYLCV antibodies (previously raised against TYLCV virus particles). ELISA and dot blot were carried out as described by Bantari and Goodwin (1985). Leaf extracts of TYLCV-infected beet and tomato plants were used as positive controls, while mock-infected Sf9 cells extracts, healthy tomato, phosphate-buffered saline (PBS) and beet leaf extracts were used as negative controls.

SDS-PAGE

To confirm expression of virus coat protein by the recombinant baculovirus, Sf9 cells were seeded into 6-well plate dishes (35 mm diameter) at a density of 10^6 cells/dish and mock-infected or infected with the recombinant vAc-TYLCV-CP at multiplicity of infection (MOI) of 10. Cells were harvested at 72 h post-infection (h.p.i.), pelleted and resuspended in 0.25 volume of $4 \times$ SDS protein sample buffer [40 mM Tris-HCl, pH 8.0, 0.4 mM EDTA, 8% SDS (w/v), 40% glycerol (v/v) and 0.004% bromophenol blue]; then the samples were boiled for 5 min. Cell extracts were loaded and run on 15% SDS polyacrylamide gel (Laemmli 1970). Gel was stained with Coomassie blue for visualization of proteins.

Results

Generation of recombinant bacmid with TYLCV-CP

Coat protein ORF (787 bp) was amplified by PCR using the genomic DNA of TYLCV (Egyptian isolate accession number AY594174.1) as a template and pair of the specific primers (Fig. 1). The obtained PCR fragment was purified, cloned into pGEM-T vector (promega) and subsequently into pFastBac-Dual under Polyhedrin promoter generating the recombinant construct pFBD-polh-TYLCV-CP.

The generated pFBD-polh-TYLCV-CP construct was transformed into DH10Bac competent *E. coli* cells that harbor a baculovirus shuttle vector (bMON14272) and a helper plasmid (pMON7142). The presence of the helper plasmid (pMON7142) supporting site-specific recombination between the generating construct pFBD-polh-TYLCV-

Fig. 1 Amplified PCR fragment of TYLCV-CP using viral genomic DNA as a template. *S* PCR fragment of 787 bp corresponding to the TYLCV-CP gene. *M* 1 Kb DNA ladder

CP and bMON14272 to generate high molecular weight bacmids harbor the TYLCV coat protein gene. Schematic representation for the construction of vAc-polh-TYLCV-CP bacmid is shown in Fig. 2. To verify successful transposition, nine clear white colonies were PCR analyzed using one pair of primers designated M13 Forward and TYLCV-Cp-R (“Materials and methods”, “PCR amplification of TYLCV-CP gene” and “Generation of the recombinant bacmid”). The PCR successfully amplified a specific band of 1400 bp in all tested colonies corresponding to the expected fragment size (data not shown). The verified recombinant bacmid was transfected and amplified in Sf9.

ELISA test

Extracts of infected insect cells with the recombinant virus harboring TYLCV-CP were reacted positively in ELISA test with antibodies raised against TYLCV particles. Extracts of TYLCV-infected tomato and beet plants were used as positive controls in ELISA as well as mock-infected extract cells, phosphate-buffered-saline (PBS), healthy tomato and beet plants as negative controls. ELISA results, diagrammed in Fig. 3, verified the antigenicity of the expressed virus coat protein by reacting positively with the TYLCV-antibodies (samples 1 and 2) comparing with negative controls (samples 3, 6, 7 and 11). Columns number 4 and 5 represent leaf extracts samples of TYLCV infected tomato, while columns number 8, 9 and 10 correspond to leaf extracts of TYLCV-infected beet plants.

Dot blot analysis

Specificity of the expressed TYLCV-CP protein using the recombinant bacmid vAc-polh-TYLCV-CP was further evaluated using dot blot analysis. The results showed that

Fig. 2 Schematic representation for the construction of vAc-polh-TYLCV-CP bacmid. The bacmid cloning vector pFBD-polh-TYLCV-CP harboring the TYLCV-CP gene under the control of Polyhedrin promoter. The relative locations of the oligonucleotides used for the

analysis of the CP locus in the generated vAc-polh-TYLCV-CP bacmid are indicated by arrows designated M13-Forward and TYLCV-CP-R. The expected PCR product sizes in vAc-polh-TYLCV-CP corresponding to the CP gene below the primer pair

Fig. 3 Detection of the CP recombinant protein expressed by vAc-polh-TYLCV-CP bacmid in Sf9 cells using 1/1000 of TYLCV antiserum by means of indirect ELISA. Bars number 1 and 2 represent extracts of infected insect cells with the vAc-polh-TYLCV-CP. Bars number 3, 6, 7 and 11 represent mock-infected extract cells, healthy tomato, Phosphate Buffer Saline (PBS) and beet leaf extracts were used as negative control, respectively. Columns number 4 and 5 represent leaf extracts of TYLCV infected tomato. Bars number 8, 9 and 10 corresponding to leaf extracts of TYLCV infected beet plants

Fig. 4 Nitrocellulose membrane dot blot analysis of the infected Sf9 cells using vAc-polh-TYLCV-CP recombinant virus against TYLCV antiserum. Spots 1 and 3 represent extracts of infected insect cells with the vAc-polh-TYLCV-CP showing positive reaction. Spots 5 and 6 leaf extracts of TYLCV infected tomato plant. Spots 7, 9 and 11 extracts of infected beet plants with TYLCV. Both infected tomato and beet plants extracts show strong signals as a positive control. No colored signals have been detected with extract of mock-infected Sf9 cells (Spot 2), healthy tomato plants (Spot 4), healthy beet plants (Spot 8) and PBS (Spot 10) as a negative control. Each reaction was duplicated for further confirmation

the TYLCV antiserum reacted positively with the recombinant CP extracts of Sf9 cells infected with vAc-polh-TYLCV-CP with strong signals as well as the TYLCV-infected tomato and beet plant extracts (as positive control). No colored signals have been detected with the extract of mock-infected cells, healthy tomato and healthy beet (negative controls) as depicted in Fig. 4.

SDS-PAGE

Infection of Sf9 cells with the recombinant baculovirus vAc-polh-TYLCV-CP produced CP that was visible on stained protein gel (Fig. 5). A protein that migrated close to the expected molecular size of ~30 kDa (the predicted size of the coat protein gene product) was observed in extracts of Sf9 cells infected with vAc-polh-TYLCV-CP at

Fig. 5 SDS-PAGE analysis of Sf9 cells infected with the recombinant virus vAc-polh-TYLCV-CP. Lanes 1, 2 represent cells lysate extracted 48 h post infection. Lanes 3, 4 represent cells extract at 72 h post infection. C Cells were mock-infected, M Page-Ruler prestained protein ladder (Thermo Fisher). TYLCV CP expressed by vAc-polh-TYLCV-CP recombinant virus at ~30 kDa is indicated

48 and 72 h.p.i. This band was absent in mock-infected Sf9 cell extracts. These results suggested the successful expression of viral coat protein using the recombinant baculovirus at the predicted protein molecular mass.

Detection of TYLCV-CP VLP in Sf9 cells by electron microscopy

Extracts of Sf9 cells infected with vAc-polh-TYLCV-CP were collected 72 h.p.i. and examined using Scanning Electron Microscope (SEM). The examination showed geminated, icosahedral virus-like particles (VLP) typical to Begomoviruses particles (20×30 nm) as indicated in Fig. 6.

Discussion

Plant virus diseases cause poor fruit yield and quality. The amount of loss usually depends on the plant host and the type of virus strain. This loss could be avoided through early detection of viral infected plants to stop virus spread within a population. In this regard, the development in molecular techniques has dramatically improved the field of diagnostics in agriculture (Sastry 2013). In the present study, the gene encoding the coat protein (CP) of TYLCV-Egyptian isolate was PCR amplified, cloned and expressed in insect cells using baculovirus expression vector system. The recombinant virus harboring CP gene was generated to examine the specificity of the expressed recombinant protein as a candidate antigen in ELISA and dot blot tests. The results showed that TYLCV-CP-based indirect ELISA was

able to detect anti-TYLCV antibody in sera raised against virus particles in all dilution samples. There was no reaction detected in all negative samples isolated from healthy tomato and beet leaf extracts. The clarified crude extract used for coating the ELISA plates did not show interference or loss in specificity suggesting no need for further purification.

The baculovirus expression system was successfully used for expression of coat protein of the Garlic Mite-borne Filamentous Virus (GarMbFV) fused with the polyhedrin gene using caterpillars as bioreactors. The study demonstrated that, fusion of the GarMbFV coat protein gene to the 3'-end of the baculovirus Polyhedrin gene was shown to produce high amounts of virus coat protein which was efficiently used as an antigen to generate virus specific antibodies (Ardisson-Araújo et al. 2013). Therefore, this approach was suggested to be used for the generation of plant virus diagnostic tools for the detection of viruses-infected plants that are present in low titers and/or in mixed infection in their plant hosts Ardisson-Araújo et al. (2013)

In the same context, the baculovirus protein expression system has been successfully used for high-yield protein expression using different Eukaryote or Prokaryote sources. A recombinant baculovirus construct expressing glycoprotein E (gE) of the Egyptian BoHV-1.1 Abu-Hammad strain (rBac/gE-AbuH) was generated and characterized. Using a simple indirect ELISA test, a recombinant glycoprotein E (gE) secreting protein was used as a coating antigen to test its capability for the detection of antibody against gE of BoHV-1. It was concluded that the developed indirect gE-ELISA is efficient candidate for inexpensive detection of anti-gE antibody with high specificity and sensitivity (El-Kholy et al. 2013). These results came in conformity with previous report that illustrates the importance of ELISA as a serological tool for diagnosis of plant viruses (Clark and Adams 1977).

The specificity of the TYLCV-CP recombinant protein was further evaluated using antiserum of TYLCV via dot blot analysis. The results showed that the TYLCV-antibodies reacted positively with extracts of vAc-polh-TYLCV-CP infected insect cells, which were used as a coating antigen, with strong signals as well as the TYLCV-infected tomato and beat plant. No colored signals have been detected with the extracts of mock-infected Sf9 cells and healthy plant samples. Different studies reported that dot blot analysis is simple, relatively inexpensive and the result can scored visually and it is relatively more sensitive and economical compared to ELISA (Rajasulochana et al. 2008; Sharma and Misra 2011). In the same context, using Bac-to-Bac baculovirus expression system, P8 protein which is the major outer capsid protein of Rice Gall Dwarf Virus (RGDV), was successfully expressed in Sf9 cells. The immunofluorescence analysis showed that P8 protein

Fig. 6 Scanning electron microscopic photograph of the TYLCV virus-like particles (VLP) detected in extracts of infected Sf9 insect cells using the recombinant virus vAc-polh-TYLCV-CP. Arrows show aggregated VLP

of RGDV formed punctuate structures in the cytoplasm of Sf9 cells. This system was used to overcome the insolubility of the expression products using prokaryotic expression system (Fan et al. 2010). Scanning electron microscope of infected insect cells using the recombinant vAc-polh-TYLCV-CP showed geminated, icosahedral virus-like particles (VLP) typical to Begomovirus particles (20 × 30 nm). We have demonstrated that TYLCV virus-like particles can be obtained by the expression of TYLCV coat proteins in insect cells. These results are in accordance with the previous data performed by Harrison (1985), who reported that TYLCV has twin (Geminate) isometric virions. The assembled VLPs showed variable shape and/or size. The possible explanation of that include: accumulation of different numbers of CP molecules participating in VLP assembly, absence of plant cellular factors required for appropriate VLP assembly and/or loss of accuracy in viral assembly resulted in the appearance of non-native structures.

However, production of plant virus-like particles (VLPs) and virus structural proteins using of baculovirus expression system has been reported to be used for members of different virus families such as: Tobacco Ringspot Virus (Singh et al. 1995), Cowpea mosaic virus (Shanks and

Lomonosoff 2000); Luteoviridae: Beet western yellows virus (BWYV) (Tian et al. 1995), Potato leaf roll virus (PLRV) (Gildow et al. 2000), Pea enation mosaic virus (PEMV) (Sivakumar et al. 2009) and the Rice dwarf virus (Hagiwara et al. 2003). This approach allowed first insight into genetic characterization of viruses especially plant viruses that are difficult to study in plants or in insect vectors (Sivakumar et al. 2009).

Conclusion

In conclusion, using of baculovirus system for protein expression of TYLCV-CP gene in Sf9 cells is simple, efficient and quick to apply. Reactivity of the produced recombinant protein with TYLCV-antibodies showed positive reaction in indirect ELISA and dot blot indicated that the baculovirus expressed TYLCV-CP was authentic and retained its antigenic properties similar to that of TYLCV particles. Consequently, the purified protein represents a promising candidate antigen for raising of high concentration of virus-CP antisera to be used in indirect CP-ELISA and dot blot as a companion diagnostic test for virus-infected plants.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in this publication.

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