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14. ABSTRACT

Polyvalent protein shells (capsids) are useful platforms for the display of molecules of interest (MOI) on their surface. The resulting polyvalent reagents can be used as efficacious prophylactic vaccines and therapeutics. The coat protein subunits of Tomato Bushy StuntVirus (TBSV) and structurally similar Norwalk viruses, when expressed in insect cells, spontaneously self assemble to form protein shells. The self-assembly of the coat protein mutants of TBSV resulted in two types of nanoparticles: small (60 subunit) and the regular size (180subunit) capsids. These protein shells (capsids) can be used for the display of 60-180 copies of peptides/proteins of the pathogens of concern .Previously, it has been shown that antibodies raised against various cytotoxins (e.g., ricin and Shiga toxin) render protection against the potential toxin attack. The proposed polyvalent reagents, which display various peptide/protein fragments of ricin would act as prophylactic vaccines of the ricin toxin by priming the immune system. We have successfully produced two polyvalent reagents displaying multiple copies of 1) 16 a.a. RTA antigenic peptide (mouse epitope) and 2) a large 188 a.a. stable RTA domain. These reagents when injected into mice generated antibodies, which readily detect the ricin toxin in the western-blot analysis. Hence, these reagents can be used as potential vaccines of the ricin toxin.

15. SUBJECT TERMS
Vaccines, Therapeutics, Cytotoxins, polyvalent display, Protein design

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INTRODUCTION & SUMMARY

The proposal titled "Creation of Polyvalent Decoys of Protein Cytotoxins as Therapeutics and Vaccines" entails displaying multiple (60-180) copies of antigenic regions of varying lengths (1-200a.a.) of the ricin toxin on the surface of viral capsids (protein shells). These polyvalent reagents (PVRs) have been shown to be efficacious prophylactic vaccines and inhibitors of the corresponding toxins (Mourez et al., 2001). In this regard, we have created the protein shells of Tomato Bushy Stunt Virus (TBSV) and Norwalk viruses by over-expressing the respective coat proteins, which self-assemble to form homogenous capsids. The 2-domain (S and P) subunit structure of these coat protein subunits facilitate the display of any proteins/peptides of interest either by replacing the C-terminal P-domains of the coat protein subunits or appending at the end of the P-domain. We have used these protein shells to generate PVRs (decoys) by appending epitopes of interest at the end of the P-domain of the TBSV capsid protein.

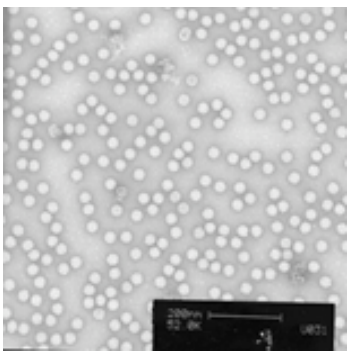
BODY OF REPORT

STATEMENT OF WORK:

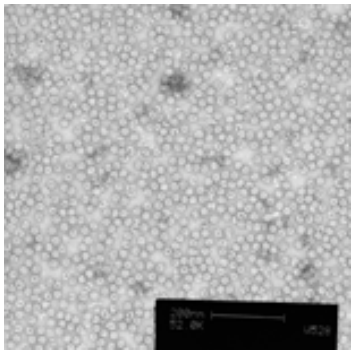
Expression and structural characterization of the polyvalent display platform of altered (mutant) capsids of Tomato Bushy Stunt Virus (TBSV) and Norwalk Viruses.

Generation of protein shells of TBSV and Sinsiro virus capsid proteins in insect cells:

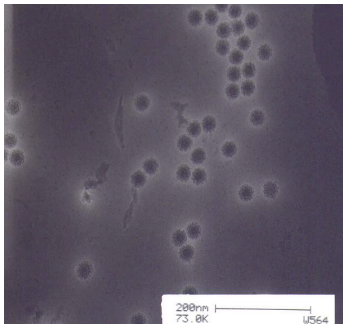
The capsid protein subunits of TBSV and Sinsiro (Norwalk) viruses have been expressed in insect cells successfully using baculovirus system. Full length (CP-FL) and a N-terminal deletion mutant (CP-NΔ52) of the TBSV capsid protein form native like (T=3) protein shells of 32nm in diameter made up of 180 protein subunits, while the longer N-terminal deletion mutants, CP-NΔ72 and CP-NΔ82 from smaller 21nm diameter



A. TBSV, CP-FL



B. TBSV, CP-NΔ72



C. Sinsiro virus, CP-FL

Figure 1. A) Electron micrographs of the 32nm nanoparticles (protein shells) assembled from the full-length TBSV coat protein. B) Smaller 21nm nanoparticles assembled from the coat protein (CP-NΔ72) with the first 72 amino acids deleted. C) 35-40nm size protein shells made of full-length coat protein of Sinsiro virus. All the samples were visualized by coating with 1% uranyl aceate stain.

(T=1) particles with 60 subunits (Figure 1). Similarly, the full-length coat protein Sinsiro virus expressed in insect cells spontaneously forms 35-40nm protein shells made of 180 subunits.

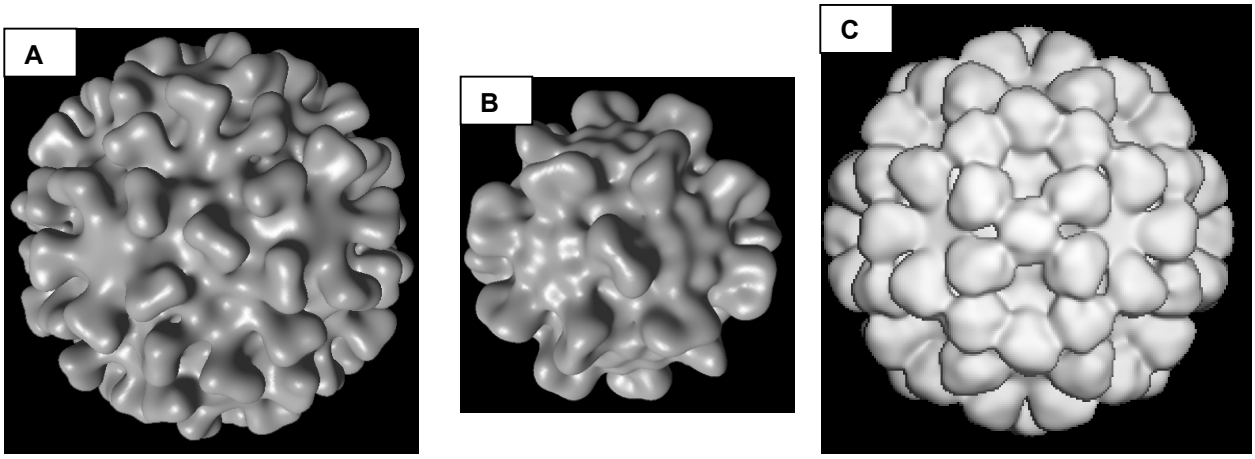


Figure 2. Cryo electron microscopy reconstructions of A) Protein shell (32nm) made of CP-NΔ52 of TBSV B) Smaller (21nm) particles made of TBSV CP-NΔ72 and C) Protein shell (~38nm) made from the full length coat protein of Sinsiro virus.

Cryo-electron microcopy and image reconstruction methods were employed to determine the structures of respective particles at about 20Å resolution (Figure 2). The above results suggest that each kind of the protein shells were made uniformly with high fidelity. Due to instability of the resulting protein shells of the Sinsiro virus, the TBSV capsids were chosen as the preferred display platforms for generating the polyvalent reagents.

Creation of polyvalent reagents (decoys) of ricin toxin by displaying RTA epitopes on TBSV capsids:

Having successfully generated two sizes of TBSV nanoparticles (protein shells) as display plat forms, the first polyvalent reagent (TBSV-RTA16) was created by genetically appending an epitope of residues 95-110 of the ricin A-chain at the C-terminal end of the TBSV coat protein. It has been shown that the above epitope of the ricin-A chain

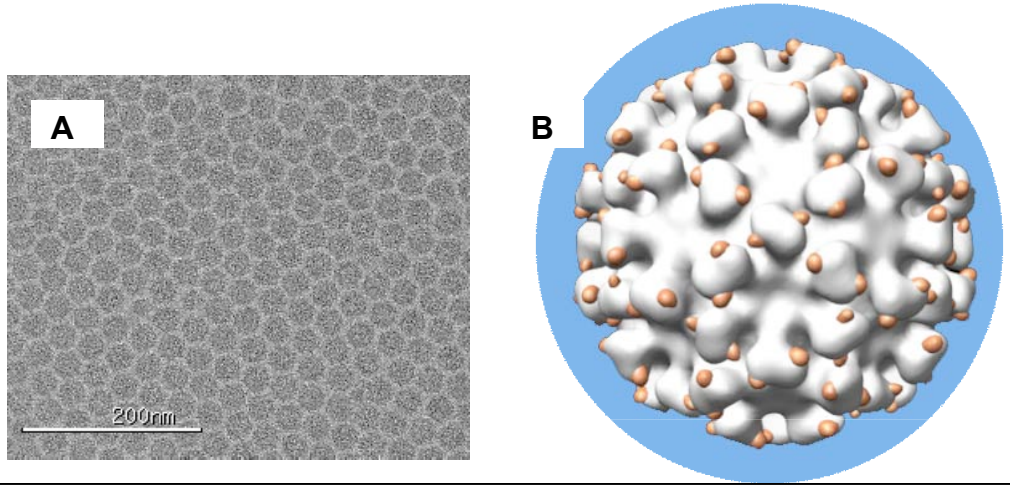


Figure 3. **A**. Electron micrograph showing the protein shells of TBSV-RTA16 with 180 copies of the RTA peptide of residues 95-110 displayed on its surface. **B**. Cryo-EM reconstruction of TBSV-RTA16 reagent (vaccine) showing the highly exposed epitopes (in gold) of the ricin toxin.

generates neutralizing antibodies that render protection against the ricin toxin (Olson et al., 2004). This novel reagent contains 180 copies of the RTA peptide displayed on the surface of TBSV capsid.

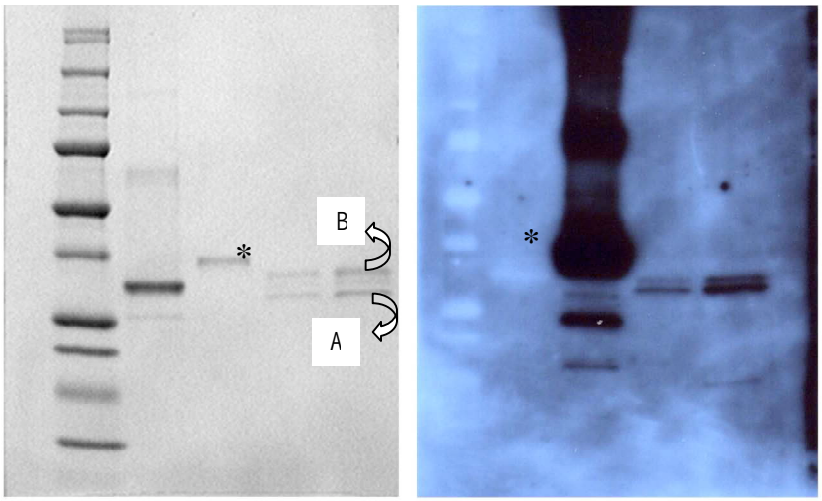


Figure 4. Detection of ricin toxin by the antibodies due to TBSV-RTA16 displaying ricin epitope. Shown on the left is the SDS page and on the right is the western blot of the proteins Lanes **1**. M.W. markers, **2**. TBSV-Δ72 (netative control), **3**. TBSV-RTA16, **4**. Ricinus communis agglutinin (RCA) as positive control. A and B chains are identified by the curled arrows, while the TBSV-RTA16 bands are identified by an asterisk.

Figure 3 shows an electron micrograph and the cryo-EM reconstruction of the TBSV-RTA16 reagent. Highly exposed ricin epitopes makes it an highly efficient reagent in producing antibodies, which detect the ricin toxin. Figure 4 shows the results that illustrate the ability of the antibodies generated in mice by the above PVR in detecting the ricin toxin. These antibodies detect the parent reagent (TBSV-RTA16) as well as the ricin toxin in the western-blot analyses. The size of the TBSV-RTA16 subunit is ~38kDa and are identified by the asterisks. Higher and lower size bands in the same lanes correspond to the multimers and cleavage products of the subunits respectively. Not surprisingly, only the ricin A-chain is detected by the antibodies due to the TSBV-RTA16 reagent. This proves that the basic methodology works in generating the antibodies, which provide protection against the ricin toxin without causing any toxic effects. These polyvalent reagents (PVRs) will be highly efficacious compared to single molecules/peptides on molar basis in generating antibodies against the ricin, hence provide greater protection against the toxin attack. Evaluation of the efficacy of the antibodies in providing protection against the ricin-toxin using the cell-intoxication experiments are underway.

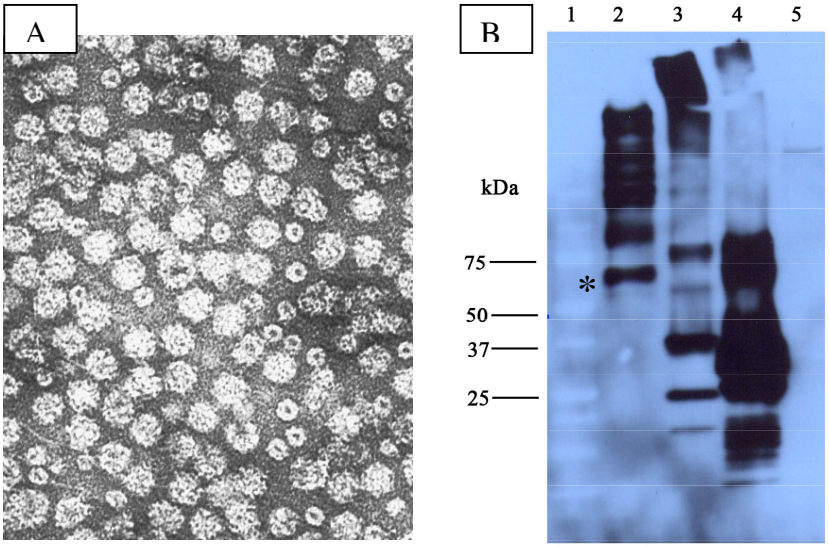


Figure 5. A. Electron micrograph of TBSV-RTA188 polyvalent reagent. Larger particles in the micrograph correspond to the TBSV-RTA188. The identity of the smaller particles is under investigation. B. Western blot analysis of the TBSV-RTA188 reagent. Lanes 1) M.W. Markers, 2) TBSV-RTA188, 3) TBSV-RTA16, 4) purified ricin toxin, 5) TBSV-Δ72 as the negative control.

We recently generated a new PVR that displays multiple copies of the stable RTA domain of 188 a.a., which by itself has been proven to be a potential vaccine candidate (Olson et al., 2004). This new reagent, namely TBSV-RTA188, was generated by appending the RTA188 domain at the end of the TBSV-subunit. Figure 5 shows the EM micrograph and a western blot analysis of the new reagent along with the TBSV-RTA16 detected using commercially available ricin antibodies. The size of the newly generated chimeric protein of TBSV-RTA188 is ~60kDa, identified by an asterisk (Figure 5B). Higher bands correspond to higher oligomers of the chimeric protein. This particular PVR appear to form smaller particles and perhaps they may contain 60 copies of the RTA domains. The actual number of RTA domains present in this PVR is currently under investigation.

KEY RESEARCH ACCOMPLISHMENTS:

1) Expression/Creation of two sizes (21nm, 32nm) of nanoparticles of TBSV capsid proteins using baculovirus system as potential display platforms for the presentation of molecules of interest by genetic modification.
2) Creation of another nanoparticle platform using Sinsiro virus (Norwalk virus) capsid protein using the baculovirus system. Due to instability of the resulting protein shells of the Sinsiro virus, the TBSV capsids were chosen as the preferred display platforms for generating the polyvalent reagents.
3) Structural characterization of the above nanoparticles using cryo-electron microscopy and image analysis.
4) Creation and characterization of polyvalent reagent (PVR), the first decoy of ricin toxin, displaying the 180 copies of the RTA peptide of residues 95-110 as a prophylactic ricin vaccine. Antibodies generated in mice using this PVR detect the native ricin toxin. Cryo-EM structural characterization of this PVR reveals the highly exposed disposition of the ricin epitopes, which is highly advantageous for efficacious vaccine.
5) Creation of a new PVR that displays multiple copies of a larger (188a.a.) and stable RTA domain. Further characterization of this reagent is underway.

CONCLUSIONS:

We have made a number of important advances in the past two years by generating polyvalent reagents (decoys) of ricin toxin as vaccines and therapeutics. In the past year we generated and structurally characterized two PVRs, which can be used as the potential vaccines of the ricin toxin. Evaluation of the efficacy of these reagents using in vitro cell intoxication experiments are currently underway.

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Olson, M. A., Carra, J. H., Roxas-Duncan, V., Wannemacher, R. W., Smith, L. A., and Millard, C. B. (2004). Finding a new vaccine in the ricin protein fold. Protein Eng Des Sel 17(4), 391-7. Epub 2004 Jun 08.

APPENDIX: Publications from W81XWH-04-2-0027

Hsu, C., Singh, P., Ochoa, W., Manayani, D. J., Manchester, M., Schneemann, A., and Reddy, V. S. (2006). Characterization of polymorphism displayed by the coat protein mutants of tomato bushy stunt virus. *Virology* **349**(1)**,** 222-9.

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Characterization of polymorphism displayed by the coat protein mutants of tomato bushy stunt virus

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Abstract

Expression of full-length and N-terminal deletion mutants of the coat protein (CP) of tomato bushy stunt virus (TBSV) using the recombinant baculovirus system resulted in spontaneously assembled virus-like particles (VLPs). Deletion of the majority of the R-domain sequence of the CP, residues 1–52 (CP-NΔ52) and 1–62 (CP-NΔ62), produced capsids similar to wild-type VLPs. Interestingly, the CP-NΔ62 mutant that retains the last 3 residues of R-domain is capable of forming both the T = 1 and T = 3 particles. However, between the two types of VLPs, formation of the T = 1 capsids appears to be preferred. Another mutant, CP-NΔ72, in which R-domain (residues 1–65) was completely removed but contains most of the β-annulus and extended arm (βA) regions exclusively formed T = 1 particles. These results suggest that as few as 3 residues (63–65) of the R-domain, which includes 2 basic amino acids together with the arm (βA) and β-annulus regions, may be sufficient for the formation of T = 3 particles. However, anywhere between 4 to 13 residues of the R-domain may be required for proper positioning of βA and β-annulus structural elements of the C-type subunits to facilitate an error free assembly of T = 3 capsids.

Keywords: Polymorphism; Virus-structure; Tombusviridae; Protein expression; Self assembly

Introduction

Tomato bushy stunt virus (TBSV) is a ~34 nm spherical plant virus that belongs to the family of Tombusviridae. Wild-type TBSV possesses a single positive-sense RNA genome of 4776 bases encapsidated into a capsid with T = 3 icosahedral symmetry, which is made of 180 copies of a single coat protein (CP) subunit of 41 kDa (Harrison et al., 1978; Olson et al., 1983). The genome of TBSV (cherry strain) was sequenced and the cDNA clones of the CP gene were generated (Hearne et al., 1990; Hillman et al., 1989). TBSV and structurally similar turnip crinkle virus (TCV) have been the subjects of structural analysis for a long period of time (Crowther and Amos, 1971; Harrison, 1971; Harrison et al., 1978; Hogle et al., 1986; Olson et al., 1983; Sorger et al., 1986; Stockley et al., 1986). The three-dimensional structures of TBSV and TCV capsids are available at high resolution (Harrison et al., 1978; Hogle et al., 1986; Olson et al., 1983). Fig. 1A shows the quaternary organization of the CP-subunits of TBSV in T = 3 capsids. The tertiary structure of the CP-subunit (Fig. 1B) is composed of three major domains: (1) the R- (RNA binding) domain, formed by the N-terminal residues (1–65) that are disordered in all the subunits, followed by the β-annulus region (residues 67–85) and an "extended arm" of residues 86-101, which are ordered only in the C-type (green) subunits (Fig. 1A); (2) the middle S- (shell) domain, a canonical β-barrel motif comprises of residues 102–268 that forms the contiguous shell of the capsid and (3) the C-terminal P- (projection) domain, made up of residues 273–388 (Figs. 1B and C). The S and P-domains are connected to each other by a four-residue hinge (269–272) (Hogle et al., 1986; Olson et al., 1983; Sorger et al., 1986).

It was shown that, in the case of turnip crinkle virus (TCV), treatment of the disassembled CP-dimers with chymotrypsin and subsequent reassembly of the proteolyzed dimers resulted in the formation of smaller (T = 1) particles (Sorger et al., 1986).

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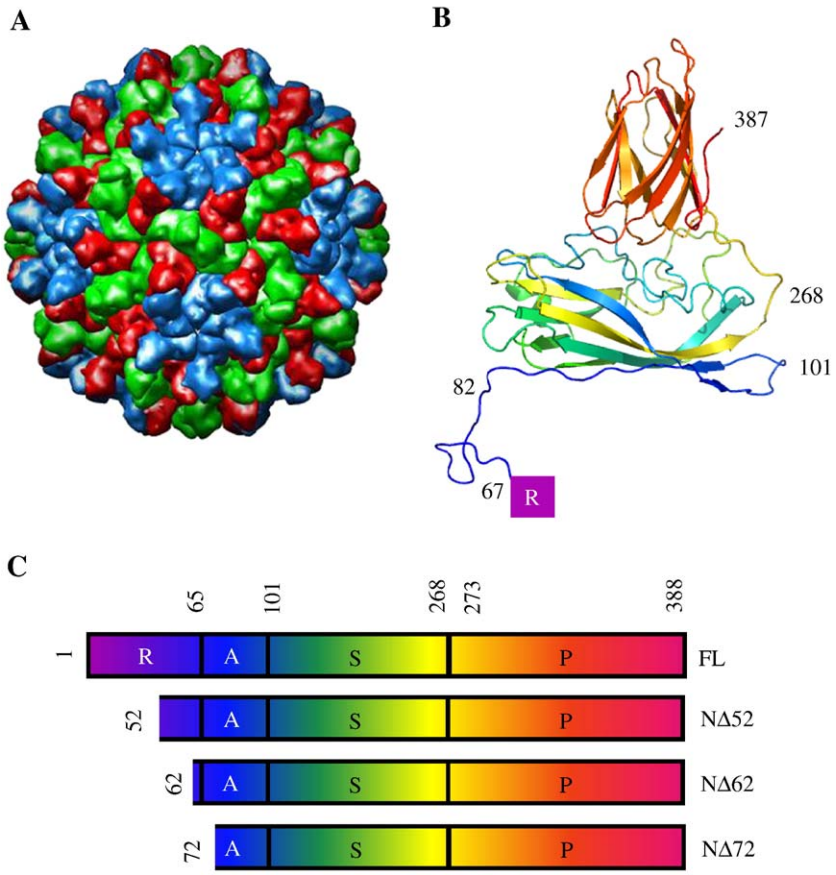

Fig. 1. Illustrations of TBSV structure and the constructions of CP deletion mutants. (A) Quaternary structure of TBSV showing the organization of (180) subunits occupying the 3 different environments (blue, red and green) in the capsid. These three color-coded environments are also commonly referred to as A (blue), B (red) and C (green) type subunits. This figure was obtained from a viral structural database, URL: http://viperdb.scripps.edu (Shepherd et al., 2006). This was generated using the program Chimera (Pettersen et al., 2004). (B) Tertiary structure (ribbon diagram) of the subunits that occupy the green (C-type) locations. A continuous color gradient (rainbow) was employed to identify different regions of the protein. Irrespective of their locations in the capsid: blue (A)/ red (B)/ green (C), the tertiary structures of all the individual subunits are nearly identical with the exception of part of the R-domain (1–65), conserved β-annulus (81–84) residues and Arm (A) (85–100) regions. Residues 1–66 are disordered in all the subunits while the residues 67–100 are selectively ordered in green (C-type) subunits. The figure was generated using the coordinates in the PDB (ID: 2TBV). This figure was generated using the program PyMol (DeLano, 2002). (C) Bar diagrams illustrating different N-terminal deletion mutants prepared and investigated in this study.

The above proteolytic cleavage at the residue 66 of the TCV-CP removed the entire R-domain and parts of the β-annulus regions from the rest of the TCV-CP subunit (Fig. 5). Hence, these regions were suggested to be responsible for forming T = 3 capsids. Even though the coat proteins of TBSV and TCV have only 21% amino acid sequence identity, they display similar subunit structure and particle (T = 3) architecture (Hogle et al., 1986). Because of this structural homology, a similar behavior of particle polymorphism was suggested for TBSV. Although the TBSV-CP gene has been manipulated in order to generate various phenotypes in plants (Hearne et al., 1990; Joelson et al., 1997; Scholthof et al., 1993), there are no reports to date on expression of the TBSV-CP gene in a heterologous expression system.

In this study, we describe the successful expression and spontaneous assembly of virus-like particles (VLPs) from the full-length and various N-terminal deletion mutants of the TBSV CP in insect cells using the baculovirus system. Furthermore, we characterized the polymorphism displayed by the N-terminal deletion mutants, in which different lengths of the R-domain and the β-annulus regions were removed. Generation of TBSV-VLPs has important advantages such as ease of exploring structure–function–assembly relationships and exploiting potential use of the VLPs as display platforms for multivalent presentation of foreign epitopes (Joelson et al., 1997).

Results and discussion

Varying lengths of TBSV-CP gene constructs were expressed in insect cells using the baculovirus expression system, and the formation of VLPs was investigated. The cDNA of the CP (p41) spanning nucleotides 2652–3815, 2805–3815, 2835–3815 and 2865–3815 corresponding to a full-length, 1–388 AA (CP-FL) and N-terminal deletion mutants 53–388 AA (CP-NΔ52), 63–388 AA (CP-NΔ62) and 73–388 AA (CP-NΔ72), respectively, were subcloned each into separate transfer vectors. The recombinant baculovirus constructs were generated using standard procedures (see Materials and methods). *Sf*-21 insect

cells infected with the respective recombinant baculovirus were lysed 3 days post-infection to harvest and analyze the expressed protein. Mock-infected and recombinant-infected cell lysates were first examined using a dot blot assay for the expression of TBSV coat protein. Cell lysates infected with each of the baculovirus constructs showed positive immunoblots indicating expression of the TBSV-CP, while the uninfected cell lysates (negative control) did not react (data not shown). Following confirmation of the expressed protein, the clarified cell lysates were subjected to ultracentrifugation through a 30% sucrose cushion. The pellets containing the partially purified VLPs were resuspended and further purified by ultracentrifugation through 10–40% sucrose gradients. Presence of faint bands visible near the middle of the gradients further confirmed the formation of VLPs. Fractionation of the gradients with continuous monitoring of absorbance at 254 nm revealed a single major peak, discounting the peak at the top of the gradient which corresponds to unassembled soluble protein and with the exception of CP-NΔ62 mutant preparation, which showed two peaks, indicating the formation of two populations of VLPs (Fig. 2A). The faster sedimenting peak nearly coincided with that of peaks from CP-FL and CP-NΔ52 preparations, while the slower sedimenting peak coincided with that of the CP-NΔ72.

SDS PAGE analysis of the purified VLPs showed that each capsid contained a single type of CP subunit (Fig. 2B). Relative to molecular mass standards, the approximate estimated masses of the expressed CP-FL, CP-NΔ52, CP-NΔ62 and CP-NΔ72 proteins were 41 kDa, 36 kDa, 35 kDa and 34 kDa, respectively. Interestingly, the antibodies raised against the wild-type TBSV failed to detect the denatured TBSV-CPs from the purified VLPs in a Western blot assay (data not shown). However, they detected the non-denatured (intact) TBSV VLPs in the dot blot assays indicating that the antibodies react with the conformational epitopes of intact TBSV capsids. Furthermore, a similar pattern was observed when antibodies raised in mice against the purified TBSV-VLPs were used. The mouse anti-TBSV antibodies did not recognize denatured TBSV antigens but reacted very strongly with native antigens in ELISA (Fig. 2C). This further confirmed that the TBSV-VLPs presumably induce only conformational antibodies. We came to know that this type of immune response is not so uncommon after we realized that the TBSV antibodies were ineffective in detecting denatured coat protein. For example, expression of human polyomavirus by recombinant baculovirus system resulted in VLPs that were able to induce only conformational antibodies when injected into rabbits. Both human and rabbit virus-specific antibodies failed to react with denatured viral antigens in dot blot and ELISA (Li et al., 2003). Joelson et al. (1997) utilized antibodies produced by hyper-immunization of rabbits with denatured TBSV particles in order to detect the chimeric virus particles displaying human immunodeficiency virus epitopes in Western blots.

Electron microscopy of negatively stained samples taken from the respective peaks (Fig. 2A) showed the presence of assembled VLPs (Figs. 3A–E). Particles formed by the CP-FL, CP-NΔ52 and the larger capsids of the CP-NΔ62 construct, have an average diameter of ~34 nm (Figs. 3A–C) consistent with the wild-type T = 3 particles of TBSV (Harrison et al., 1978;

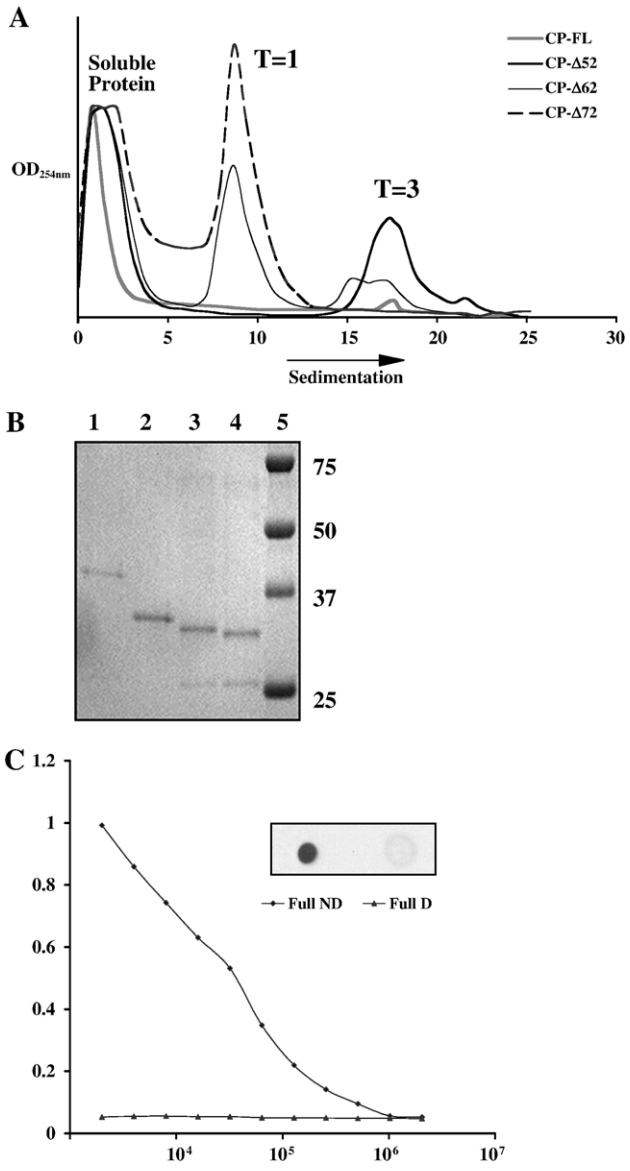

Fig. 2. Biophysical and biochemical characterization of the VLPs of TBSV. (A) Plot showing the UV absorbance profiles of CP-FL, CP-NΔ52 and CP-NΔ62 and CP-NΔ72 is shown. (B) SDS-PAGE analyses of TBSV-VLPs showing the Mr of the corresponding CP-subunits, lanes 1: CP-FL, 2: CP-NΔ52, 3: CP-NΔ62, 4: CP-NΔ72 and 5: molecular weight markers. Tris-Glycine (10–20%) gels (Invitrogen, Carlsbad, CA) were used for all the SDS-PAGE analysis. (C) ELISA assay showing the selective detection of non-denatured coat proteins (●) by the mouse anti-TBSV antibodies over denatured coat protein (▲). Inset shows the corresponding dot blot. In both cases, antigens of CP-FL VLPs were used.

Olson et al., 1983). The smaller particles, ~21 nm in diameter, generated by the CP-NΔ62 and CP-NΔ72 proteins most likely correspond to T = 1 particles. Even though, the CP-NΔ62 forms both types of particles, the formation of the T = 1 particles appears to be more efficient than that of the T = 3 capsids. Similar kinds of smaller (T = 1) particles were obtained earlier by the proteolysis and subsequent reassembly of subunit dimers of turnip crinkle virus (TCV), which is structurally analogous to TBSV (Sorger et al., 1986). TEM examination suggested that a majority of VLPs were not empty (Figs. 3A–E), with the exception of T = 3 particles from CP-NΔ62, where a number of

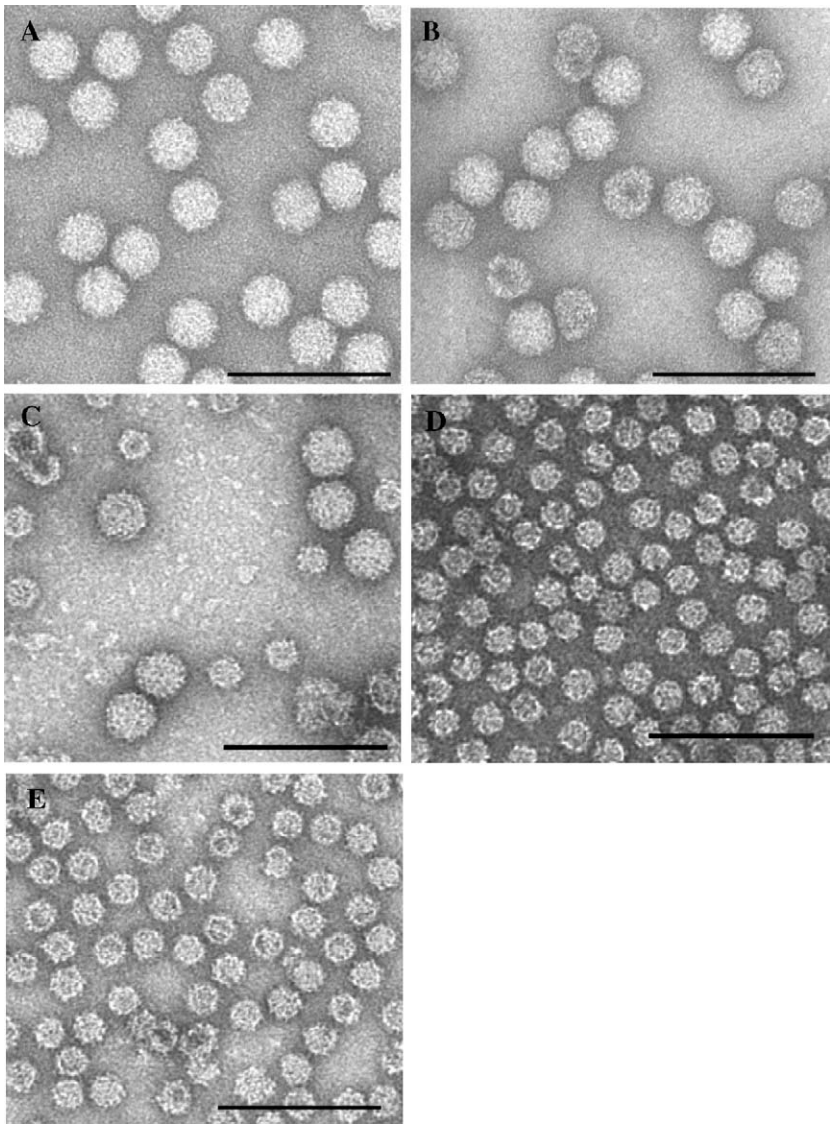

Fig. 3. Electron micrographs of TBSV particles. Micrographs showing the formation of particles of different TBSV CP-phenotypes. (A) CP-FL, (B) CP-NΔ52, (C) CP-NΔ62 (T = 3), (D) CP-NΔ62 (T = 1) and (E) CP-NΔ72. Scale bar = 100 nm.

stain-permeated broken particles are seen (Fig. 3C). The observation of full particles is not surprising, as it was suggested that viral or heterologous RNA induced the assembly of TCV capsids (Sorger et al., 1986; Stockley et al., 1986). The packaged RNAs were extracted from the respective particles and analyzed by agarose gel electrophoresis. Fig. 4 shows that the packaged nucleic acids contain a collection of RNAs ranging from 100–1900 bp. Since there is no specific band corresponding to the size of full-length mRNAs (~1100 bases), we did not further characterize the origin of the packaged RNA as it is not the scope of the current work. However, it is quite possible that the packaged RNAs contain a mixture of degraded mRNAs and cellular RNAs as was observed in similar investigations (Agrawal and Johnson, 1995; Dong et al., 1998; Lokesh et al., 2002; Schneemann and Marshall, 1998; Taylor et al., 2002). It is interesting to note that even though all or most of the N-terminal basic residues were deleted, the T = 1 particles formed by the CP-NΔ62 and CP-NΔ72 proteins appear to package RNA (Fig. 4).

The TBSV-CP of 388 amino acids (AA) is 37 residues longer than the TCV-CP of 351 AAs. Alignment of the two coat protein (CP) sequences resulted in 291AA pairs aligned with 21% sequence identity (Fig. 5). The majority of the additional (21) residues in TBSV are located in the R-domain, upstream of the β-annulus forming region of residues 67–85. Since the last basic amino acid upstream of the conserved β-annulus region of TBSV is found at the residue 65, it was assumed that the R-domain comprises residues 1–65. Therefore, a larger number (65) of amino acids are involved in forming the R-domain of TBSV compared to that of TCV (52 AAs). Interestingly, the number of basic AAs is identical (12) in the R-domains of both TBSV and TCV with the last basic AA located at positions 65 and 57, respectively, before the start of the conserved β-annulus forming region. The N-terminal deletion mutants CP-NΔ52, CP-NΔ62 and CP-NΔ72 of TBSV contain 7, 2 and 0 basic residues respectively upstream of the β-annulus region. The corresponding VLPs of CP-NΔ52 (T = 3), CP-NΔ62 (T = 3 and

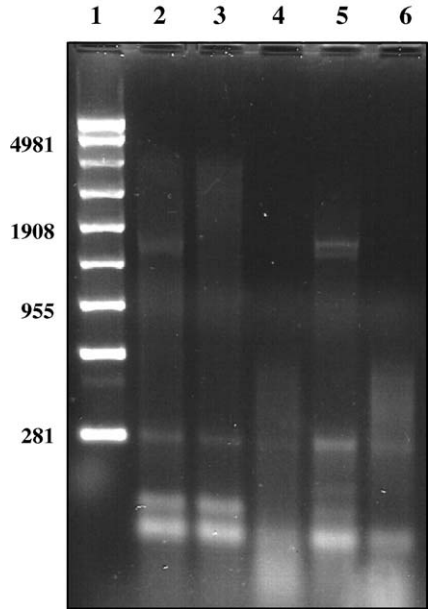

Fig. 4. Agarose gel electrophoresis of RNA. Gel electrophoretic profile of RNA extracted from different TBSV-CP phenotypes. Lanes 1: RNA marker (Promega, Madison, WI), few sizes in bases are shown on the left side, 2: CP-FL, 3: CP-NΔ52, 4: CP-NΔ62 (T = 3), 5: CP-NΔ62 (T = 1) and 6: CP-NΔ72.

T = 1) and CP-NΔ72 (T = 1) suggest that the length and possibly the number of positively charged residues in the R-domain may play a role in the formation of T = 3 capsids. The efficient formation of the T = 3 capsids by the CP-NΔ52 suggests that the last 13 residues of R-domain that include 7 of the total 12 basic residues in the R-domain are adequate for the formation of T = 3 capsids. These observations coupled with the results on CP-NΔ62, which forms both type of particles (T = 3 and T = 1), we hypothesize that as few as 3 residues of the R-domain, beyond the β-annulus forming region, maybe sufficient to form T = 3 particles of TBSV. These results clearly demonstrate that only a few residues (3–13) of the 65 residue R-domain are required for the formation of T = 3 capsids and conceivably, the same holds true for TCV capsids as well.

Similar results have been reported on Sesbania mosaic virus (SeMV), where deletion of 36 or 65 residues from the N-terminus of CP resulted in T = 1 particles, compared to the full-length CP and CP-NΔ22 yielded T = 3 particles (Lokesh et al., 2002; Sangita et al., 2004; Satheshkumar et al., 2004). Since the presence of the arm region by itself or together with a segment forming the β-annulus region was not sufficient to produce larger (T = 3) particles, Savithri and coworkers suggested the need for at least part of the R-domain, also known as the arginine rich motif for the formation of T = 3 particles (Lokesh

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TBSV_CP MAMVKRNNNTGMIPVSTKQLLALGAAAGATALQGFVKNNGMAIVEGAVDL   50
TCV_CP  -----------MENDPRVRKFASDGAQWAIKWQ----------KKGWSTL   29
                   *   .  : :* ..*  *   *           :*   *

TBSV_CP TKRAYKAVRRRGGKKQQMINHVGGTGGAIMAPVAVTRQLVGSKPKFTGRT  100
TCV_CP  TSRQKQTARAAMGIKLSPVAQPVQKVTRLSAPVALAYREVSTQPRVSTAR   79
        *.*  ::.*   * * . : :   .   : ****:: : *.::*:.:

TBSV_CP SGSVTVTHREYLSQVNNSTGFQVNGGIVGNLLQLNPLNGTLFSWLPAIAS  150
TCV_CP  DG-ITRSGSELITTLKKNTDTEP----KYTTAVLNPSEPGTFNQLIKEAA  124
        .* :* :  * :: :::.*. :       .   *** :   *. *   *:

TBSV_CP NFDQYTFNSVVLHYVPLCSTTEVGRVAIYFDKDSEDPEPADRVELANYSV  200
TCV_CP  QYEKYRFTSLRFRYSPMSPSTTGGKVALAFDRDAAKPPPNDLASLYNIEG  174
        ::::* *.*: ::* *:..:*  *:**: **:*: .* * * ..* * .

TBSV_CP LKETAPWAEAMLRVPTDKIKRFCDDSSTSDHKLIDLGQLGIATYGGAGTN  250
TCV_CP  CVSSVPWTGFILTVPTDSTDRFVADG-ISDPKLVDFGKLIMATYGQGAND  223
          .:.**:  :* ****. .**  *.  ** **:*:*:* :**** ...:

TBSV_CP AVGDIFISYSVTLYFPQPTNTLLSTRRLDLAGALVTASGPGYLLVSRTAT  300
TCV_CP  AAQLGEVRVEYTVQLKNRTGSTSDAQIGDFAGVKD-----GPRLVSWSKT  268
        *.    :  . *: : : *.:  .::  *:**.       *  *** : *

TBSV_CP VLTMTFRATGTFVISGTYRCLTATTLGLAGGVNVNSITVVDNIGTDSAFF  350
TCV_CP  KGTAGWEHDCHFLGTGNFSLTLFYEKAPVSGLENADASDFSVLGEAAAGS  318
          *  :.    *: :*.:         . ..*::  . : .. :*  :*

TBSV_CP INCTVSNLPSVVTFTSTGITSATVHCVRATRQNDVSLI  388
TCV_CP  VQWAG----VKVAERGQGVKMVTTEEQPKGKLQALRI-  351
        :: :       *:  . *:. .*..     : : : :
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Fig. 5. Sequence alignment. The CP sequence of TBSV (Swiss-Prot-ID: P11689) aligned with that of Turnip Crinkle Virus (TCV) (Swiss_Prot_ID: P06663). The stars indicate the locations of identical residues, while ':' and '.' correspond to functionally homologous and related residues, respectively. The entire R-domain including the Arginine rich basic amino acids of TBSV is shown in gray color.

et al., 2002; Sangita et al., 2004). Prior to that based on the proteolysis and reassembly experiments on TCV coat protein dimers, it was suggested that the R-domain and the arm may be required for the formation of T = 3 capsids (Sorger et al., 1986). However, because of the limited nature of the latter study, the extent of the R-domain and the arm regions of the CP required for the formation of T = 3 capsids was not very clear, particularly in the family of Tombusviridae. Our results reported in this study, especially on the CP-NΔ62 phenotype, suggest that even 3 residues of the R-domain containing two basic residues, upstream of the β-annulus forming region are sufficient to produce T = 3 capsids. However, efficient formation of T = 3 capsids by the CP-NΔ52 suggests that a slightly larger R-domain (13 residues) with more basic residues (7) may be necessary for the "error free assembly" of T = 3 capsids. The majority of viruses have evolved over long time periods, hence the complete R-domain may be functionally important for an efficient and selective packaging of viral genomes, delivery, replication and other aspects of the viral life cycle in plants. Our results taken together with the previous reports on SeMV (Lokesh et al., 2002; Sangita et al., 2004; Satheshkumar et al., 2004) suggest a common theme for the formation of T = 3 capsids, namely that the peptide arm, also known as βA, alone or together with β-annulus forming region, is not sufficient for the formation of T = 3 capsids.

Recent studies on the specific role of basic amino acids and β-annulus regions of SeMV or lack of it in the formation T = 3 particles point to the requirement on the length of polypeptide chain in these regions rather than the AA composition (Satheshkumar et al., 2005). They also point out that origin of the formation of β-annulus structure, which is stabilized primarily by main chain–main chain hydrogen bonds, is a consequence of capsid assembly rather than the result of specific AA sequence motif. Even though some sequences might have evolved to preferentially form such structures, they may not be absolutely essential. In other words, capsid assembly may facilitate the formation of such structures if there are sufficient number of amino acids present upstream of β-annulus forming region. This "sufficient" number of AAs might depend on a particular CP subunit forming the T = 3 capsid. However, the basic amino acids present in the R-domain are required for encapsidation of viral or cellular RNA. This hypothesis is in agreement with our results in this study as well as Savithri and Co-workers (Lokesh et al., 2002; Satheshkumar et al., 2005; Satheshkumar et al., 2004). The requirement of the R-domain in terms of its length, composition and the resulting protein–nucleic acid interactions presumably is a "means to the end" for correctly positioning the β-annulus and βA in place thereby facilitating the efficient formation of the T = 3 capsids and RNA encapsidation.

Materials and methods

Cell culture

Spodoptera frugiperda cells (line IPLB-Sf21) were grown at 27 °C in TC100 medium (Invitrogen, Carlsbad, CA) supplemented with 0.35 g of $NaHCO_3$ per liter 2.6 g of tryptose broth per liter, 2 mM L-glutamine (final concentration), 100 U of penicillin per mL and 100 μg of streptomycin per mL and 10% heat-inactivated fetal bovine serum (Schneemann et al., 1993). Cultures were maintained as monolayers in screw-capped plastic flasks or as suspensions in 1-L spinner flasks.

Generation of recombinant baculoviruses expressing TBSV coat protein

The cDNA clone of TBSV-CP in plasmid pTCTBcp (Hearne et al., 1990; Hillman et al., 1989) was a generous gift of Drs. T.J. Morris and F. Qu (University of Nebraska, Lincoln, Nebraska). In order to generate recombinant baculovirus, the full-length or mutant TBSV-CP sequence was inserted into a baculovirus transfer vector, pBacPAK9 (BD Biosciences-Clontech, Mountain View, CA). TBSV-CP full-length or truncated sequences were amplified by PCR from the pTCTBcp plasmid. To facilitate cloning of inserts into the multiple cloning site of pBacPAK9, a *Bam*HI and a *Xho*I restriction site sequences were added to the 5′ and 3′ ends of the CP sequences, respectively. The 5′ primers are BTSTART (to amplify full-length CP) 5′ GGATCCATGGCAATGGTAAAG AGAAACAACAAC3′, BT52 (to amplify CP-NΔ52) 5′CGGGATCCATGAAAA-GAGCGT ACAAAGCAGTC3′, BT62 (to amplify CP-NΔ62) 5′GGATCCATGGGAGGTAA GAACAGCAGATG3′ and BT70 (to amplify CP-NΔ72) 5′CGGGATCCATGGTA GGTGGTACAGGTGGTGC3′. The XTRev reverse primer, 5′ CTCGAGCTAAATTAG AGAAACATCATTCTGTCG3′ used in all PCR reactions, anneals to the C-terminus of the TBSV-CP and also adds a TAG stop codon to the end of all CP sequence inserts. All PCR reactions were performed using Expand High Fidelity Polymerase® (Roche, Indianapolis, IN). Amplified PCR products were first cloned into PCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) and were sequenced and aligned with the published TBSV-CP sequence (GenBank accession: M21958). Confirmed constructs digested with *Bam*HI and *Xho*I restriction enzymes were subcloned into respective sites of pBacPAK9 transfer vector. Recombinant baculoviruses expressing different TBSV-CPs were generated by co-transfecting each of the pBacPAK9 plasmid constructs with linearized baculovirus DNA in pBacPAK6, according to manufacturer's instructions (BD Biosciences Clontech). Cultures containing recombinant baculovirus were plaque purified and amplified to obtain pure stocks of each construct with high viral titers.

Protein expression and purification of VLPs

Sf-21 cells were plated at a density of 8×10^6 cells per 10 cm tissue culture dish and were infected with stocks of the respective recombinant baculoviruses at an MOI of 0.5–2 pfu per cell. Cells and supernatant were harvested 72–96 h post-infection. Nonidet P-40 was added to a final concentration of 1% (v/v) and incubated on ice for 10 min to lyse the cells. Cell debris was pelleted by centrifugation at 13,800×*g* for 10 min at 4 °C in a JA17 rotor (Beckman, Fullerton, CA). The VLPs in the

supernatant were pelleted through a 30% sucrose (wt/wt) cushion by ultracentrifugation at 184,048 × *g* for 2.5 h in a 50.2 Ti rotor (Beckman) at 4 °C. Pellets were then resuspended in the sodium acetate buffer (100 mM $C_2H_3NaO_2$, 50 mM NaCl, 10 mM $CaCl_2$ and pH 5.5) overnight at 4 °C. Insoluble material in the resuspended pellet was removed by centrifugation at 12, 000 × *g* for 10 min in a micro-centrifuge. The supernatant was analyzed by SDS-PAGE analysis using 10–20% Tris-Glycine gel and stained with Simply Blue stain (Invitrogen, Carlsbad, CA) as well as dot blot analysis. To further purify VLPs, the supernatant was loaded onto 10–40% sucrose gradients (wt/wt) made in sodium acetate buffer and centrifuged at 197, 500 × *g* for 2 h in a SW-41 rotor (Beckman). VLPs were harvested from gradients by puncturing the sides of the tubes with a needle and aspirating the bands using a syringe or by ISCO gradient fractionator (Teledyne, Los Angeles, CA) at 0.75 mL/min and 0.5 min per fraction.

Immunodetection of TBSV coat protein

Both cell lysates collected from Sf-21 cells infected with recombinant baculovirus and purified VLPs were assayed by Western dot blot for detecting the expression of TBSV protein. Samples were applied as dots onto a nitrocellulose membrane and allowed to air dry for 5 min at room temperature. A positive control consisted of native TBSV particle antigens, a gift of Dr. H.B. Scholthof (Texas A and M University, College Station, Texas). The membrane was then incubated at room temperature (RT) for 1 h in a blocking buffer made of 1% non-fat dry milk in phosphate-buffered saline (PBS). After blocking, the membrane was treated for 1 h at RT with rabbit anti-TBSV antibody (ATCC, Manassas, VA) diluted-1000 fold in the blocking buffer containing 0.05% Tween 20. The membrane was then washed twice for 15 min at RT with washing buffer made of 0.05% Tween in PBS. Afterwards, the membrane was incubated for 30 min at RT in blocking buffer containing a 2000-fold dilution of anti-rabbit IgG conjugated to horseradish peroxidase (Amersham, Piscataway, NJ). Following incubation, the membrane was washed four times in washing buffer. Antigen–antibody complexes were visualized by incubation of blot in Super Signal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

Pooled sera from three mice, hyper-immunized with purified TBSV-CP(NΔ52) VLPs were examined in a enzyme linked immunosorbent assay (ELISA). Approximately 1 μg/well of TBSV-VLP antigens either native or denatured by heating (10 min at 90 °C) in mixture of SDS and β-mercaptoethanol was coated on ELISA plates (Immunolon II, Dynatech Laboratories Inc., Chantilly, VA) in a coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate buffer, pH 9.6) overnight. The wells were blocked by 1% bovine serum albumin in a tris-buffered saline (TBS, 50 mM Tris–HCl, 140 mM saline, pH 8.0) for 1 h at RT. Each well was exposed to serial dilutions of hyper-immune serum for 1 h at RT. After the wells were treated with wash buffer (TBST,0.25% tween-20 in TBS) three times, they were exposed to horse radish peroxidase-labeled anti-mouse antibody (Pierce Biotechnology, Rockford, IL) diluted (1:10,000) in wash buffer for 1 h at RT. The wells were washed three times with wash buffer and the bound antibodies were detected with a TMB-peroxidase kit (Kirkegaard and Perrry Laboratories Inc., Gaithesburg, MD). Substrate reaction was stopped with 1N hydrochloric acid and the absorbance at 450 nm was determined using a Versamax® microplate reader (Molecular Devices, Sunnyvale, CA).

Extraction of RNA from VLPs and agarose gel electrophoresis

RNA was extracted from purified VLPs with acidic phenol-chloroform after the addition of sodium chloride and SDS to a final concentration of 0.2 M and 0.1%, respectively. RNA in the aqueous fraction was precipitated with 0.3 M sodium acetate (pH 5.2) and 1 μg of glycogen as a carrier. RNA pellet was washed in 70% ethanol, vacuum dried and resuspended in nuclease-free water. Approximately 2 μg of RNA was loaded on 1% agarose gel made in MOPs buffer (0.02 M MOPS, pH 7.0, 2 mM sodium acetate, 1 mM EDTA, pH8.0) with formaldehyde to eliminate secondary structures. The RNA gels were subjected to gel electrophoresis in MOPs buffer with formaldehyde at 150 V for 1.5 h. RNA bands were visualized by staining with ethidium bromide.

Electron microscopy

Carbon-coated, copper grids were glow discharged in the presence of amyl acetate immediately before loading samples. Grids were placed in 10 μL of VLP samples for 1 min, washed three times in sterile filtered water and stained in 10 μL drops of 1% uranyl acetate three times allowing the grids to soak in the last drop of stain for 2 min. After drying grids, samples were examined under an electron microscope (Phillips CM100).

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