Intermolecular Interactions of *Chrysanthemum virus* B Coat Protein: Implications for Capsid Assembly

Lakhmir Singh · Vipin Hallan · Aijaz A. Zaidi

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Abstract *Chrysanthemum virus* B coat protein constitutes the viral capsid which, besides other functions, encapsulates and protects the viral nucleic acid. We have demonstrated homotypic interaction of the coat protein subunits, essentially important for dimer formation, which is the first step during capsid assembly in vivo and in vitro. Interaction capacity of full length and truncated protein has been investigated and important regions have been identified through protein–protein interaction in yeast and by co-immunoprecipitation assays. Complete coat protein was found to interact strongly with similar subunits. Constructs with 102 amino acids from the N-terminal and 64 amino acids from C-terminal were found to be inconsequential for dimer formation as they did not show any interaction with similar subunits or with full length protein when analysed for β -galactosidase or histidine prototrophy. Results suggest that the region of 98–184 amino acids from the middle plays an important role in the process, probably without the involvement of N- and C- terminals.

Keywords *Chrysanthemum virus* B · Capsid · Dimer · Protein–protein

Introduction

Chrysanthemum the “queen of the East” is a popular perennial flowering plant of (Family Asteraceae) commercial importance, widely grown in different parts of India over acreage of 4,000 hectares and rest of the tropical

world. *Chrysanthemum* is next to rose in importance among the flower crops in the world. It ranks 3rd in world among the cut flowers [24]. The plant is commonly propagated by vegetative means, and this practice allows viral and viroid pathogens, once established in the plants, to be perpetuated from generation to generation. Several viruses and viroids have been reported in *chrysanthemum*, including *Chrysanthemum virus* B (CVB) [21], *Tomato aspermy virus* [19], *Tomato spotted wilt virus* [18], *Chrysanthemum stem necrosis virus* [29], *Chrysanthemum chlorotic mottle viroid* [22] and *Chrysanthemum stunt viroid* [9].

Chrysanthemums world-wide experience a high incidence of CVB: a species in the *Carlavirus* genus of the family *Flexiviridae* [30]. CVB is one of the major pathogens of *chrysanthemum*. This virus is known to occur worldwide (wherever *chrysanthemums* are grown), is a quarantine pathogen and a potential threat to the floriculture industry [25]. CVB has slightly flexuous rod shaped particle of 680×12 nm and is transmitted by aphid *Myzus persicae* in a non-persistent manner and is also sap transmissible. *Carlavirus* genome comprises of single stranded monopartite, plus sense ssRNA molecule ~ 8.8 kb in size with a poly (A) tail at 3'-terminus and a cap structure or a monophosphate at their 5'-terminus [4, 5, 15, 31]. The genomic RNA is encapsidated by a single type of coat protein (CP) with molecular weight 34 kDa. Genome of CVB consists of six open reading frames (ORF), only ORF5 codes for the capsid protein, the building blocks of the viral particle. Viral capsules are the protein shells that surround and protect the viral genome. In our lab we have sequenced the CP of 32 geographical isolates which show three major lineages of the virus in India [25]. Complete genome of four isolates was established. Antisera based diagnostic kit was developed by expressing the CP gene of the virus to facilitate mass

L. Singh · V. Hallan (✉) · A. A. Zaidi
Plant Virology Lab, Institute of Himalayan Bioresource
Technology, Palampur, Himachal Pradesh 176061, India
e-mail: rnaivi@gmail.com

screening of the planting material [26]. Virus transmission from one plant to the next (through vectors or other means) is dependent upon successful and correct assembly of the virion. Capsid assembly is a relatively complex process that involves a large but specific number of protein subunits interacting with each other and viral nucleic acid. Process involves repeated interaction of well-defined protein subunits in a highly specific process [20].

A functional CP is essential for host plant infection and insect transmission of viruses, so basic research into mechanism of protein polymerization and virus assembly suggest that inhibition of protein–protein interaction necessary for their assembly is a valid strategy for combating viruses. Till date there is no information on self interaction of the CP of this virus. Therefore, molecular basis of homotypic interaction (dimer formation) will be the starting point for a better understanding of the capsid assembly. This was a prerequisite to develop control strategies against the virus based on the concept of pathogen derived resistance. The detailed pathways by which CP subunits dimerize and polymerize, and the mechanisms by which CVB assembles to form capsid in the presence or absence of nucleic acid, are largely unknown. In the present study we have analyzed homotypic interaction of the CP at the level of dimer formation. Intermolecular interaction was detected *in vivo* and *in vitro* using the yeast two-hybrid system (Y2H) and co-immunoprecipitation (Co-IP) respectively.

Material and Methods

To check homotypic interaction of the CP of CVB, Y2H protein-protein interaction assay was used [3]. Complete CP gene was amplified by polymerase chain reaction (PCR) using HF2-Advantage PCR system (Clontech, Takara Bio, Japan) and a pair of primer containing the *SmaI-NdeI* restriction sites to facilitate in frame cloning in the Y2H plasmids GAL4 DNA-binding domain (DNA-BD) pGBKT7 and activation domain (AD) pGADT7 vector (Clontech, Takara Bio, Japan). pGBKT7 contains c-Myc epitope tag, while pGADT7 contains HA epitope tag which can be used to verify protein–protein interactions *in vitro* by Co-IP using antibodies against these tags. To map interacting domains of the protein, nine deletion constructs were generated using primer pairs corresponding to various regions of the protein so that the role of N, C terminal and middle (M) region may be analysed (Fig. 1).

PCR amplicons corresponding to truncated CP were cloned in pGBKT7 and pGADT7 vectors. Three independent clones for each fragment were checked by sequencing. Plasmids (AD and BD) with various parts of CP, 1–315 aa complete CP, truncated region 1–184 aa, 1–102 aa,

98–315 aa, 98–256 aa, 98–184 aa, 180–315 aa, 180–256 aa, 251–315 aa encode the Gal4 DNA binding domain and Gal4 activation domain fusion proteins. Yeast host strains Y187 (MATa, *ura3–52*, *his3–200*, *ade2–101*, *trp1–901*, *leu2–3*, 112, *gal4Δ*, *met[−]*, *gal80Δ*) [8] and AH109 (MATa, *trp1–901*, *leu2–3*, *ura3–52*, *his3–200*, *gal4Δ*, *gal80Δ*) [13] were used for transformation (Clontech, Takara Bio, Japan). Yeast transformation was achieved by Lithium acetate method [6, 10, 12, 23]. Yeast competent cells were prepared and suspended in a LiAc solution with both plasmid DNA to be cotransformed (0.5 μg each), along with sheared 100 μg herring testes DNA as carrier. Polyethylene glycol (PEG) with the appropriate amount of LiAc was added and the mixture of DNA and yeast were incubated at 30°C. After the incubation, DMSO was added and the cells were subjected to heat shock at 42°C. Cells were then plated on minimal media containing 2% glucose, 2% agar, 1.7 g/l Yeast Nitrogen Base (YNB) free of amino acids, 1.4 g/l amino acid mix lacking Leucine, Tryptophan (-LT) and 5 g/l ammonium sulphate as nitrogen supplement. Colonies cotransformed with recombinant pGADT7 and pGBKT7 plasmids were streaked on media lacking Leucine and Tryptophan (-LT). Cotransformed yeast colonies were checked for the presence of fragments by PCR using vector specific primers. Cotransformants in yeast host strain Y187 and AH109 were analysed for β-galactosidase assay (x-gal overlay) and histidine prototrophy (-LHT) assays, respectively. To check leaky His expression in AH109, 3-amino-1,2,4-triazole (3-AT) was added to the media to the level of 10 mM. Only clones containing interacting regions were expected to show β-galactosidase activity and to grow on histidine lacking media. All fragments cloned in pGADT7 and pGBKT7 were checked for transcriptional activation by co-transformation with pGBKT7-Lam and empty pGADT7, respectively.

After detecting protein interactions through Y2H screen, *in vitro* Co-IP (biochemical) assay was performed using antibodies against either c-Myc or HA epitope tag to confirm true protein interactions. For Co-IP, *in vivo* interacting clones in pGADT7/HA and pGBKT7/Myc were translated *in vitro* using the TNT quick coupled transcription/translation system (Promega, USA) labeled with ³⁵S-Met methionine following manufacturer's instruction. *In vitro* translated HA tagged and Myc tagged fragments were co-immunoprecipitated using the Matchmaker Co-IP kit (Clontech, Takara Bio, Japan), where *in vitro* translated proteins were incubated with either c-Myc monoclonal antibody or HA-tag polyclonal antibody along with protein A beads following the manufacturer's instructions. The antibody–protein complexes were then resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to autoradiography.

Fig. 1 A schematic representation of different deletion constructs of CVB CP used in the study. Full length CP encodes 315 aa, nine deletion constructs (A–I) were generated. The positions of amino acids encoded by each construct are indicated with line

Table 1 Interaction between full length CP and different CP deletion constructs along with positive and negative controls

Co-transformed clones and their description	Assay results	
	β -galactosidase (Y187)	His prototrophy (AH109)
1–315 aa (AD)/1–315 aa (BD)	+	+
1–315 aa(AD)/1–256 aa (BD)	+	+
1–315 aa (AD)/98–184 aa (BD)	+	+
1–315 aa (AD)/98–315 aa (BD)	+	+
1–315 aa (AD)/98–256 aa (BD)	+	+
1–184 aa (AD)/1–184 aa (BD)	++	++
1–184 aa (AD)/1–315 aa (BD)	+	+
1–184 aa (AD)/1–256 aa (BD)	+	+
98–315 aa (AD)/180–315 aa (BD)	+	+
98–315 aa (AD)/251–315 aa (BD)	–	–
180–256 aa (AD)/251–315 aa (BD)	–	–
180–315 aa (AD)/251–315 aa (BD)	–	–
1–256 aa (AD)/98–315 aa (BD)	+	+
1–256 aa(AD)/251–315 aa (BD)	–	–
1–184 aa (AD)/1–102 aa (BD)	–	–
1–184 aa (AD)/98–184 aa (BD)	++	++
98–184 aa (AD)/98–184 aa (BD)	++	++
1–184 aa (AD)/98–256 aa (BD)	+	+
1–184 aa (AD)/98–315 aa (BD)	+	+
1–184 aa (AD)/180–256 aa (BD)	+	+
1–184 aa (AD)/180–315 aa (BD)	+	+
pGADT7-T/pGBKT7-53: Positive control	+++	+++
pGADT7-T/pGBKT7-Lam: Negative control	–	–
pGBKT7: Negative experimental control	–	–
pGADT7: Negative experimental control	–	–
1–315 (BD)/pGADT7: Transcriptional activation control	–	–
1–315 (AD)/pGBKT7-Lam: Transcriptional activation control	–	–

Each construct has been indicated by the amino acid position cloned in AD and BD vector. Y187 strain cotransformed with plasmids were checked for interaction by β -galactosidase assay. AH109 strain cotransformed with AD and BD plasmids were checked for interaction by their ability to grow on medium lacking histidine (-LTH with 10 mM AT). Positive and negative controls have been shown +, Weak interaction; ++, mild interaction; +++, strong interaction

Results and Discussion

A total of 109 co-transformations were carried out using the different fragments cloned in pGADT7 and pGBKT7 in various combinations to check the interaction between different parts of the CP. Y2H analysis showed homotypic interaction of the complete CP, mean complete CP cloned in pGADT7 showed interaction with the complete CP

cloned in pGBKT7. Results of some interaction assay have been shown in Table 1. Amino acids 1–184 in AD showed interaction with complete CP in BD, 1–256 aa, 1–184 aa in BD, 98–184 aa in BD, 98–256 aa in BD and 98–315 aa in BD. Complete CP does not interact with 102 aa from N-terminus, while it interacts with protein expressed from a clone representing 1–184 aa from the N-terminus and middle region of the CP (Figs. 2; 3). Therefore, the 86 aa

Fig. 2 β -galactosidase assay of positively interacting clones, which were transformed with the following combination: 1: 1–315 (AD)/1–315 (BD), 2: 1–256 (AD)/1–315 (BD), 3: 1–184 (AD)/98–184 (BD), 4: 98–184 (AD)/98–184; +ve and –ve denotes the positive and negative control for β -galactosidase assay

Fig. 3 Nutrient based assay of AH109 cell growth on histidine deficient medium and 10 mM 3-AT at different dilutions transformed with different combination of AD/BD plasmids

Fig. 4 Co-IP assay shows that 98–184 aa (BD) co-immunoprecipitate with 1–184 aa (AD). The proteins were transcribed and translated in vitro from 98–184 aa (c-Myc) and 1–184 aa (HA) and assayed by co-IP. Lane 1: (1–184AD) + (98–184BD) + c-Myc antibody, Lanes 2, 3: (98–184BD) + c-Myc antibody, Lane 4: (1–184AD) + (98–184BD) + HA-Tag antibody

region spanning from 98–184 is extremely important which also shows strong interaction with itself. It is also worth noting that the clones expressing C-terminus 251–315 aa do not interact with either itself or full CP. Results suggest that interaction probably takes place between the mid portions of CP without involvement of N and C-terminus. Truncated version of CP, N-terminus (1–102) and C-terminus (251–315) failed to interact with itself and with wild type CP. Positive interactions were counted on the basis of the β -galactosidase assay and ability of the interactor to grow on medium lacking histidine (–LTH with 10 mM AT).

Two hybrid results were confirmed by Co-IP analysis. It was seen that in vitro translated protein from in vivo interacting constructs containing 98–184 aa and 1–184 aa reacted strongly. They were examined whether they precipitated together with c-Myc antibodies or with HA antibodies. We found that incubation of c-Myc monoclonal antibodies with mixture of in vitro translated protein from c-Myc tagged 98–184 aa and HA-tagged 1–184 aa resulted in precipitation of both proteins using the protein A beads. In the same manner, addition of HA polyclonal antibodies to the mixture of in vitro translated protein HA-tagged 1–184 aa and c-Myc-tagged 98–184 aa resulted in Co-IP of these two proteins (Fig. 4).

Results of both assays showed the homotypic interaction of the CP of the CVB. First, interaction of full length CP was studied and our results demonstrate the homotypic interaction of the protein analyzed by β -galactosidase assay and the ability of the transformed yeast cell to grow on medium lacking histidine. Further, deletion analysis showed that the constructs containing the region of CP from 98–184 aa show interaction with full length CP, itself and different deletion constructs containing the amino acid

98–184 which suggests that interactions take place in region spanning 98–184 aa. This region is a must in both the interaction partners as absence of the regions from one partner renders “no-interaction” (Table 1). This is evident from the interaction analysis results of clone 1–102 aa which failed to either self-interacts or interacts with a complete CP. Further, 98–184 aa region was successful in such an interaction. It was observed that 108–184 aa region contained a “WD” sequence which is an important component of F-box proteins [14]. However, F-box proteins usually have seven repeats of the motif whereas the CVB CP was found to contain WD and DW motifs only once. As the underlying common function of all WD-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions, its tempting to speculate that WD motif is actually responsible for interaction of the two CP units. It was also noticed that F-box proteins form seven β -anti-parallel sheets due to seven WD repeats. Therefore it may be hypothesized that with the WD sequence two subunits form an anti-parallel β sheets during dimer formation.

Many enveloped ssRNA viruses such as human immunodeficiency virus, *Moloney murine leukemia virus*, *Hepatitis C virus*, and *Sendai virus* [1, 11, 16, 17] show the phenomenon of multimerization of nucleocapsid proteins. Capsid proteins play a pivotal role in the structural organization of the viral life cycle. The primary function of viral core proteins, is assumed to provide a protective shell for the viral genome inside. No information is available about the mechanism of how CVB CP assembles in a capsid structure, the details of self interaction might provide basis for a better understanding of capsid formation. At molecular level by analyzing the deletion mutants by Y2H and Co-IP, we have identified a region of the protein (98–184 aa) involved in homotypic interaction. While a truncated protein lacking the 98–184 aa lost the capability of forming homodimer. A deletion construct lacking the N and C terminal region is still able to participate in homotypic interaction. The observation made in the study can be an important step toward understanding the capsid formation in case of carlavirus group, as has been suggested in case of other viruses, a head-to-tail organization of the homotypic interaction of *Tomato yellow leaf curl virus* [7] and TSWV N protein where N-terminal and a C-terminal regions of the protein are important for capsid assembly [28]. In case of *Alfalfa mosaic virus* CP, two separate regions in the C-terminus were found critical for dimer formation and assembly [2, 27]. C-terminus of *Cowpea chlorotic mottle virus* CP is essential for dimer formation and particle assembly [32].

Virus capsid assembles thorough the repeated interaction CP subunits in a highly specific process. Basic research into the mechanism of protein polymerization and virus assembly suggests that inhibition of protein–protein interaction

necessary for their assembly is a valid strategy for combating viruses [20]. Homotypic interaction assays provide the first clue to the understanding and elucidation of the molecular mechanism(s) involved in CVB (carlavirus) capsule assembly and will be helpful in designing strategies for resistance through inhibition of dimer formation.

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