Virology 409 (2011) 156–162

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

βC1 encoded by tomato yellow leaf curl China betasatellite forms multimeric complexes *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 21 June 2010
Returned to author for revision
14 September 2010
Accepted 5 October 2010
Available online 28 October 2010

Keywords:
βC1
TYLCCNB
Multimerization
Granular bodies
Betasatellite

ABSTRACT

The βC1 protein encoded by betasatellites associated with begomoviruses is multi-functional. To investigate its properties, the βC1 protein encoded by tomato yellow leaf curl China betasatellite (TYLCCNB) was expressed in *Escherichia coli* and analyzed for its ability to self-interaction. The βC1 protein formed large soluble multimeric complexes *in vitro* and *in vivo*. Mutations that prevented formation of multimeric complexes *in vitro*, also prevented formation of granular bodies *in vivo*, suggesting that granular bodies resulted from βC1 oligomerization. Similarly, βC1 mutants unable to form complexes also did not induce typical symptoms in plants when expressed from a Potato virus X (PVX) vector, suggesting that βC1 self-interaction was required for symptom induction *in planta*. Deletion analysis revealed that amino acid sequences spanning two predicted α-helices at the C-terminal end of the protein were important in multimerization.

Introduction

The genus *Begomovirus* in the family *Geminiviridae* consists of a group of plant viruses which caused enormous economic losses to crops worldwide (Mansoor et al., 2006). The typical begomovirus genome consists of two molecules of circular ssDNA, known as DNA-A and DNA-B, and each is approximately 2.6–2.8 kb in length. However, the majority of begomoviruses in the Old World consist of only a single genomic component, homologous to DNA-A of bipartite begomoviruses (Stanley et al., 2005). Betasatellites are small circular ssDNA molecules (about 1.3 kb) associated with the majority of monopartite begomoviruses (Briddon and Stanley, 2006). Betasatellites are dependent on their helper begomoviruses for replication, encapsidation and movement within plants (Briddon et al., 2003; Briddon and Stanley, 2006) but are required, in many cases, by their helper begomoviruses for symptom induction in hosts from which they were isolated (Briddon et al., 2001; Jose and Usha, 2003; Li et al., 2005; Saunders et al., 2000). All betasatellite molecules encode an approx. 13.5 kDa protein, known as βC1, in the complementary-sense orientation, which is a pathogenicity (symptom) determinant (Cui et al., 2004; Guo et al., 2008; Saeed et al., 2005; Saunders et al., 2004). βC1 can also suppress host cell RNA silencing (Cui et al., 2005; Gopal et al., 2007; Sharma et al., 2010) and may complement movement of DNA-A component of bipartite begomoviruses in the absence of DNA-B (Saeed et al., 2007).

The βC1 encoded by tomato yellow leaf curl China betasatellite (TYLCCNB) is a symptom determinant, a suppressor of RNA silencing and it interacts with the protein Asymmetric Leaves 1 (AS1) from *Arabidopsis* to alter leaf development (Cui et al., 2004, 2005; Yang et al., 2008). Despite these findings, biochemical and structural properties of TYLCCNB βC1 have not been investigated. We report here that βC1 forms large multimeric complexes *in vitro* and granular bodies *in vivo*, and that multimerization is necessary for βC1 to induce typical disease symptoms in plants.

Results

Y10βC1 forms large multimeric complexes in vitro

A vector for expression of the βC1 gene of TYLCCNB isolate Y10 (Y10βC1) fused to an N-terminal His tag in *Escherichia coli* was produced (Fig. 1A, upper panel) and the protein was purified by affinity chromatography. During gel filtration, only a single peak at an elution volume of 48.6 mL (void volume = 45 mL) was detected (Fig. 1A, middle panel). The calculated MW of the peak is more than 1000 kDa. The peak elute was then subsequently analyzed by SDS-PAGE and western blot. A single band with MW of about 16 kDa, the size predicted for monomeric His-Y10βC1, was detected (Fig. 1A, bottom panel). The result indicated that Y10βC1 formed large multimeric complexes *in vitro*.

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doi:10.1016/j.virol.2010.10.007

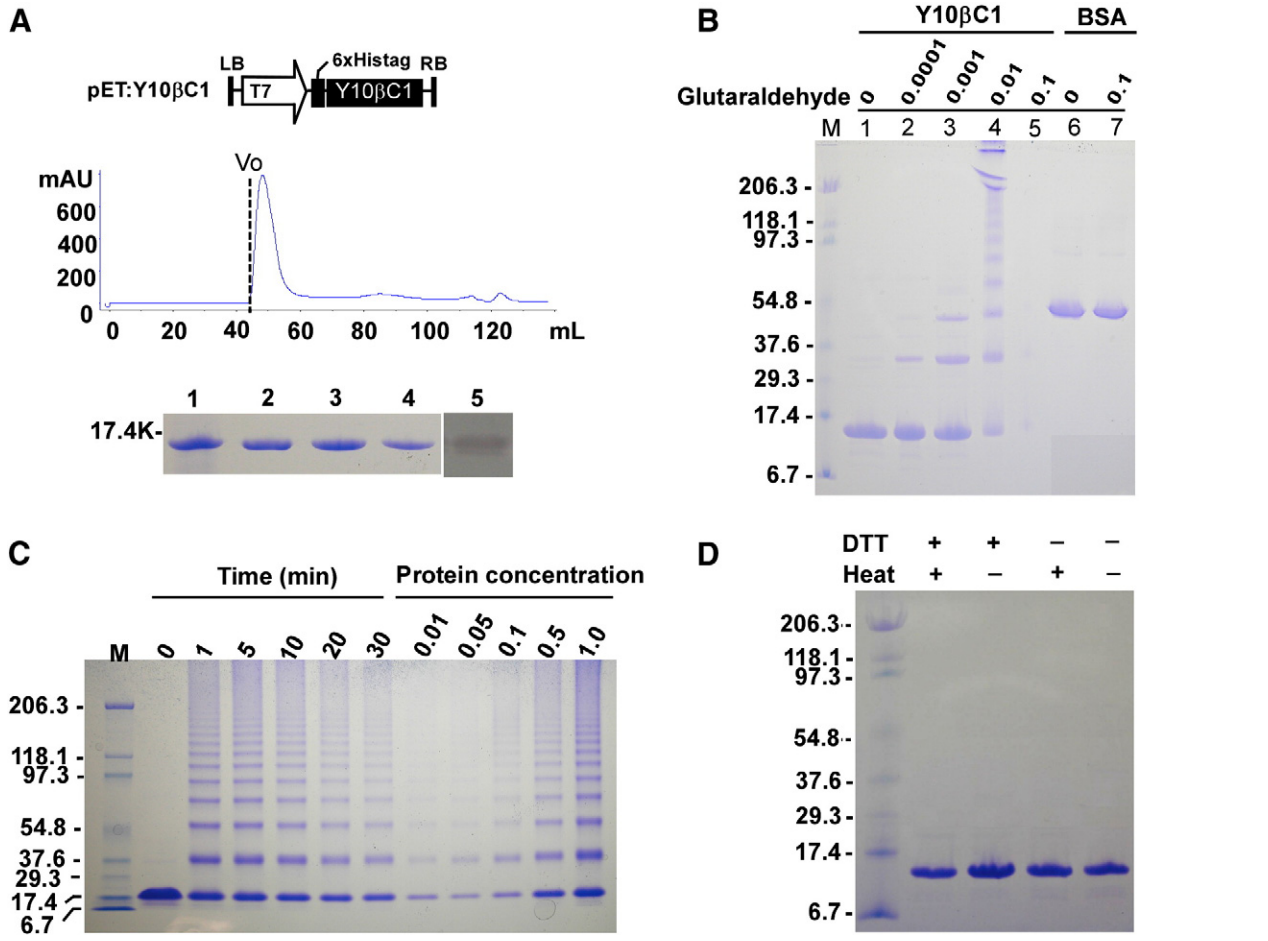

Fig. 1. Y10βC1 forms large soluble multimeric complexes *in vitro*. (A) Estimation of the apparent molecular weight of His-Y10βC1 by gel filtration. The construct containing the full coding sequence of Y10βC1 (pET:Y10βC1) is shown in the upper panel. Left and right borders of the binary vector are indicated as LB and RB, respectively. The positions of the T7 promoter and Histag are shown. The elution profile of His-Y10βC1 is shown in the middle panel. Absorption at 280 nm is indicated on the left, elution volume is indicated at the bottom and the void volume (Vo) is shown by a dashed line. SDS-PAGE and Western blot of peak elutes from gel filtration are given in the bottom panel. Lane 1 to 4, elution of 46, 47, 48 and 49 mL, respectively. Lane 5, Western blot of peak elution (48 mL) using monoclonal antibodies against the Y10βC1 protein. (B) Cross-linking of Y10βC1 protein (lanes 1 to 5) or bovine serum albumin (BSA, lanes 6 and 7) with glutaraldehyde. Final concentration of glutaraldehyde in each reaction is indicated at the top. The products of cross-linking were electrophoresed on 8–25% PhastGel. (C) Effects of incubation time and protein concentration on cross-linking. Reaction time (minute) and protein concentration (mg/mL) are shown at the top of the gel. (D) Effect of DTT and heat on the multimerization of Y10βC1. Purified Y10βC1 protein was either untreated (–) or treated (+) with DTT and/or heating to 100 °C for 5 min prior to electrophoresis.

Cross-linking is a widely used method for characterizing protein self-interaction (Nadeau and Carlson, 2002). Purified Y10βC1 was incubated with various concentrations of glutaraldehyde. After cross-linking, products were analyzed on SDS-PAGE gels. After incubation with low concentrations of glutaraldehyde (0.0001% and 0.001%), two higher molecular mass forms were present, their MWs corresponded to dimeric and trimeric Y10βC1. Following treatment with 0.01% glutaraldehyde, a ladder-like pattern of bands with a regular spacing was observed. At an even higher concentration of glutaraldehyde (0.1%), Y10βC1 failed to enter into the gel during electrophoresis, remaining in the wells, suggesting that cross-linked protein complex was too large to enter the gel matrix. In contrast, BSA migrated at a constant position on SDS-PAGE gels, even after treatment with 0.1% glutaraldehyde (Fig. 1B).

Incubating time and protein concentration have been reported to affect cross-linking results (Rudolph et al., 1997). To avoid this possibility, purified Y10βC1 protein was diluted approximately 100 times (to about 0.01 mg/mL protein) and then treated with 0.01% glutaraldehyde for various time periods and a series of protein dilutions were treated with glutaraldehyde for a set time period (20 min). The same ladder-like pattern was observed for all treatments (Fig. 1C), indicating that cross-linking products were not resulted from high protein concentrations or extended incubation times.

The predicted sequence of Y10βC1 contains a cysteine residue at amino acid position 68. To rule out that multimerization is mediated by formation of intermolecular disulfide bonds, purified Y10βC1 was analyzed by SDS-PAGE under reducing and non-reducing conditions. Thus, the protein samples were prepared in sample loading buffer with or without reducing agent DTT and with or without heat treatment at 100 °C for 5 min prior to electrophoresis. The results showed that a single band of approx. 16 kDa, representing Y10βC1 monomer, was present in protein samples following all treatments (Fig. 1D). This indicated that βC1 multimerization was not due to intermolecular disulfide bonds.

Y10βC1 forms granular bodies in vivo

To eliminate that the observed multimeric complexes represent aggregations of misfolded Y10βC1, bimolecular fluorescence complementation (BiFC) assay was used to evaluate the ability of Y10βC1 to self-interact *in vivo*. No YFP signal was observed in leaves co-infiltrated with empty vectors (pYN1 and p2YC), nor in leaves inoculated with pYN:Y10βC1 and p2YC or pYN1 and p2YC:10βC1

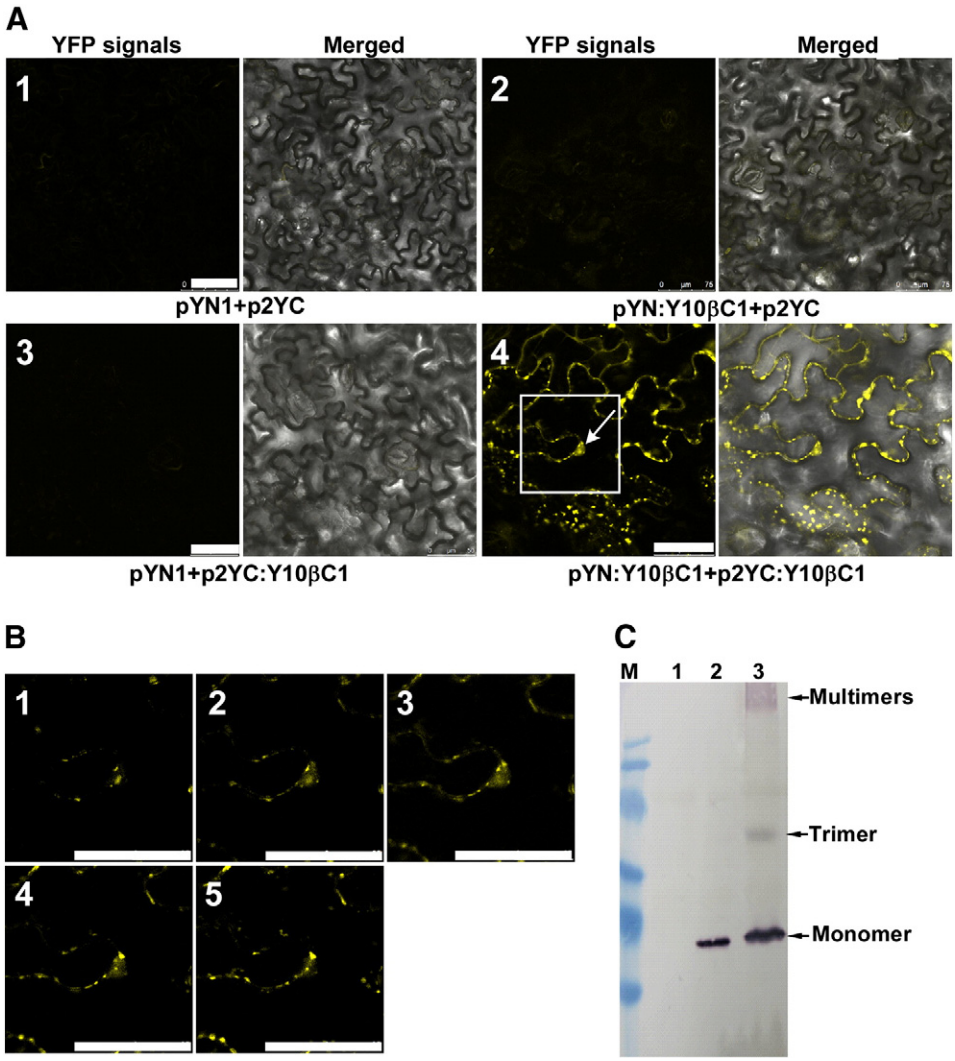

Fig. 2. Y10βC1 forms granular bodies *in vivo*. (A) Visualization of Y10βC1 self-interactions in *N. benthamiana* epidermal cells by BiFC assay. *N. benthamiana* leaves were co-agroinoculated with pYN1 and p2YC empty vectors (1), pYN:Y10βC1 and p2YC (2), pYN1 and p2YC:Y10βC1 (3) or pYN:Y10βC1 and p2YC:Y10βC1 (4). In panel 4 a nucleus is indicated by the arrow. (B) Serial vertical optical sections of the boxed region in (A4). Sections (1 to 5) were taken at a 1 μm intervals. Scale bars represent 50 μm in each case. (C) *In vivo* cross-linking of Y10βC1. Total proteins were extracted from *Nicotiana benthamiana* leaves agroinfiltrated with empty pJL TRBO vector (lane 1), pTRBO:Y10βC1 without cross-linking (lane 2) or pTRBO:Y10βC1 cross-linked with glutaraldehyde (lane 3). The position of monomer, trimer and multimer of Y10βC1 were indicated. M, protein molecular weight marker (Bio-Rad).

(Fig. 2A, 1 to 3). However, strong granular YFP fluorescence was detected in the cytoplasm of cells agroinfiltrated with pYN:Y10βC1 and p2YC:Y10βC1, indicating intermolecular interaction between the two Y10βC1 fusion proteins that restored the ability of split YFP to fluoresce. The sizes of yellow spots in the cytoplasm were highly variable, ranging from 0.5 to 10 μm in diameter (Fig. 2A, 4). Consistent with our earlier studies (Cui et al., 2005), YFP signal was also detected in nuclei. However, only a diffusive YFP signal was observed in nuclei, although many yellow granular bodies were observed around the nucleus (Fig. 2B). These results showed that Y10βC1 may also form homo-multimers *in vivo*. Oligomeric state of Y10βC1 was also investigated by *in vivo* cross-linking. Intact leaves of *N. benthamiana* plants infected with a Tobacco mosaic virus (TMV) based vector expressing native Y10βC1 were treated with glutaraldehyde to cross-link interacting proteins. After the reaction, total proteins were isolated, run on SDS-PAGE gels and detected on western blots with a monoclonal antibody against Y10βC1. In addition to a band migrating at the size predicted for monomeric Y10βC1, several higher MW bands were detected from the leaves treated with glutaraldehyde (Fig. 2C). These results clearly showed that Y10βC1 also formed multimeric complexes *in vivo*.

Y10βC1 self-association is mediated by two putative α-helixes

To identify possible sequence motifs responsible for multimerization, the secondary structure of Y10βC1 was predicted from its amino acid sequence using PredictProtein Server (Rost and Liu, 2003). The structure of Y10βC1 is predicted to contain four β-strands and two α-helices. Three of the four predicted β-strands are located within the N-terminal half of Y10βC1 (amino acids 1–50), whereas the two α-helices and the fourth β-strand are located within the C-terminal half of Y10βC1 (amino acids 61–118) (Fig. 3A). Based on this prediction, Y10βC1 was arbitrarily split into two domains, the N-terminal domain (NTD, encoding amino acids 1–60) and the C-terminal domain (CTD, encoding amino acids 61–118). These gene fragments were introduced into pYN1 and p2YC, respectively. BiFC assays showed that NTD was not competent for interaction with full length Y10βC1, with itself or with CTD, since no fluorescence was detected in *N. benthamiana* leaves agroinoculated with pYN:NTD and p2YC:Y10βC1, pYN:NTD and p2YC:NTD or pYN:NTD and p2YC:CTD (Fig. 3B, 1 to 3). In contrast, strong YFP signals were detected in leaf cells agroinoculated with pYN:CTD and p2YC:CTD or pYN:CTD and p2YC:Y10βC1 (Fig. 3B, 4 and 5), showing that CTD was able to interact with itself and with Y10βC1.

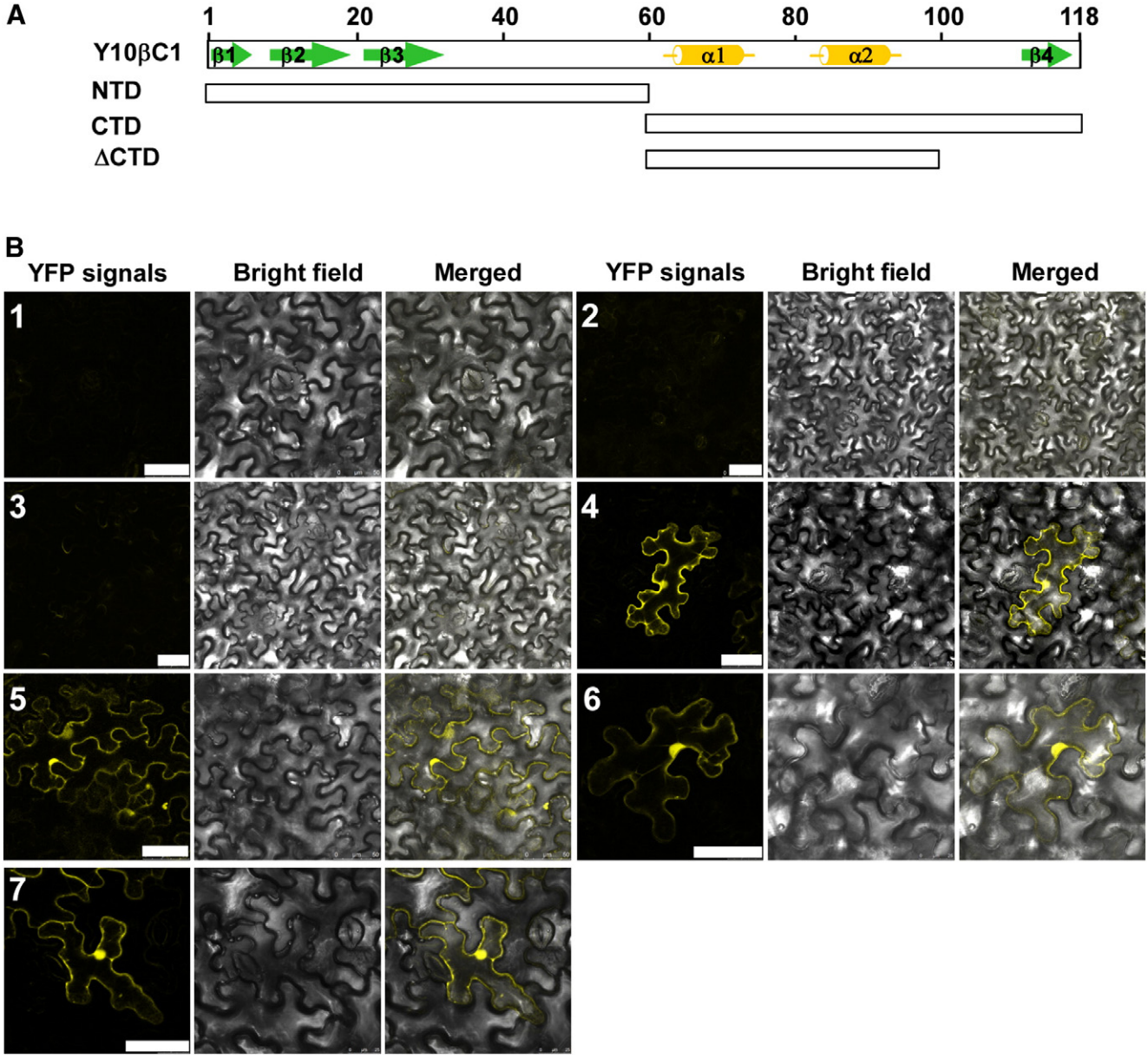

Fig. 3. Determination of the self-association domain of Y10βC1. (A) Schematic illustration of predicted structural elements of Y10βC1 and the N- and C-terminal truncated mutants of Y10βC1. Predicted β-strands and α-helices are shown as green arrows and yellow bars, respectively. (B) Visualization of interactions of Y10βC1 deletion mutants in *N. benthamiana* epidermal cells by BiFC assay. *N. benthamiana* leaves were co-agroinoculated with pYN:NTD and p2YC:NTD (1), pYN:NTD and p2YC:Y10βC1 (2), pYN:NTD and p2YC:CTD (3), pYN:CTD and p2YC:CTD (4), pYN:CTD and p2YC:Y10βC1 (5), pYN:ΔCTD and p2YC:ΔCTD (6) and pYN:ΔCTD and p2YC:Y10βC1 (7). The scale bars represent 50 μm.

Since the most prominent features of CTD are two predicted α-helices, we suspected the two α-helices may mediate multimerization. Consequently a third construct, for expression of only the sequence predicted to contain the two α-helices (ΔCTD, expressing amino acids 60–100 of Y10βC1), was produced for analysis using BiFC. ΔCTD remained capable of interacting with CTD and with wild type Y10βC1 in BiFC assays (Fig. 3B, 6 and 7). This finding showed that multimerization of Y10βC1 was mediated by amino acids sequences that span the two α-helices located between amino acids 60 and 100.

Multimerization is necessary for Y10βC1 to induce symptom in N. benthamiana

We have previously shown that transgenic expression of Y10βC1 with an N-terminal influenza virus hemagglutinin tag (HA:βC1) in *Arabidopsis* induced virus-like symptoms (upward-curled leaves), whereas expression of Y10βC1 with a C-terminal Myc tag (βC1:Myc) led to phenotypically normal plants (Yang et al., 2008). To eliminate that these results were due to the type of fusion partner used, native non-tagged Y10βC1 (construct pGR:Y10βC1), as well as Y10βC1 with N- or C-terminal maltose binding protein (MBP) tags (constructs pGR:MBP-Y10βC1 and pGR:Y10βC1-MBP, respectively), were expressed in *N. benthamiana* plants using a Potato virus X (PVX) vector (Jones et al., 1999). All *N. benthamiana* seedlings inoculated with pGR:Y10βC1 developed severe virus-like symptoms, including leaf curling, twisted stems and stunting, at 20 dpi (Fig. 4A, 1). *N. benthamiana* seedlings inoculated with pGR:MBP-Y10βC1 also developed virus-like symptoms that were indistinguishable from those induced by expression of Y10βC1 (Fig. 4A, 2). In contrast, *N. benthamiana* seedlings inoculated with pGR:Y10βC1-MBP did not develop severe symptoms (Fig. 4A, 3), these plants showed very mild vein yellowing symptoms typical of PVX infection of *N. benthamiana* (Fig. 4A, 4). We then expressed N-

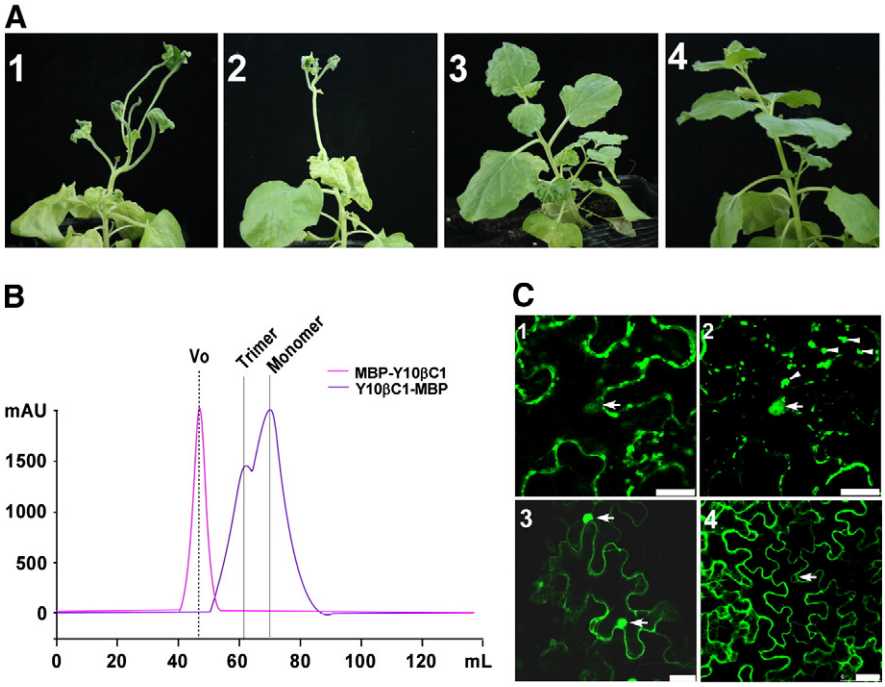

Fig. 4. The relationship between symptom induction and the formation of granular bodies. (A) Symptom of *N. benthamiana* plants infected with PVX expression constructs following agroinoculated with pGR:Y10βC1 (1), pGR:MBP-Y10βC1 (2), pGR:Y10βC1-MBP (3) or the PVX vector with no insert (4, as negative control). (B) Elution profile of MBP-Y10βC1 (pink curve) and Y10βC1-MBP (violet curve). Absorption at 280 nm, the void volume (Vo) and calculated MWs of the peaks are labeled. (C) GFP fluorescence in *N. benthamiana* leaves agroinfiltrated with, pCHF3:Y10βC1-GFP (1 and 2), pCHF3:GFP-Y10βC1 (3) and pCHF3:GFP (4). The representative nuclei are marked by arrows in each case and granular bodies inside the cytoplasm are marked with arrow heads. Images (1 and 2) represent the same cells at different confocal planes. All bars represent 25 μm.

and C-terminal MBP tagged Y10βC1 (MBP-Y10βC1 and Y10βC1-MBP) in *E. coli*. MBP-Y10βC1 eluted near void volume in gel filtration indicating that Y10βC1 with a N-terminal tag is capable of forming high order multimers *in vitro*, whereas Y10βC1-MBP eluted as two peaks with the main peak corresponding to the size predicted for monomeric and minior peak for trimeric (Fig. 4B). This indicated that forming high order multimers were necessary for Y10βC1 to induce symptom in *N. benthamiana*.

To investigate the effects of N- and C-terminal tagging on subcellular localization of βC1, constructs with fusions of green fluorescence protein (GFP) were produced in binary expression vectors (pCHF3:GFP-Y10βC1 and pCHF3:Y10βC1-GFP). GFP signal was observed in both the cytoplasm and the nucleus in *N. benthamiana* leaves infiltrated with pCHF3:GFP-Y10βC1 and pCHF3:Y10βC1-GFP (Fig. 4C, 1–3), but granular GFP signal was only observed at the cytoplasm in *N. benthamiana* leaves infiltrated with pCHF3:GFP-Y10βC1 (Fig. 4C, 1 and 2). As a control, a diffuse signal was detected in both the cytoplasm and the nuclei of cells of leaves infiltrated with pCHF3:Y10βC1-GFP or pCHF3:GFP (Fig. 4C, 4). The results indicated that N- and C-terminal tagging had no effect on subcellular localization of Y10βC1, but only N-terminal tagging Y10βC1 (GFP-Y10βC1) formed granular bodies *in vivo*.

Discussion

βC1 is a multi-functional protein encoded by betasatellites that are associated with the majority of monopartite begomoviruses (Briddon and Stanley, 2006). To determine the active form of the protein *in planta*, the βC1 protein of TYLCCNB was analyzed by gel filtration, chemical cross-linking and BiFC assays. The results showed that Y10βC1 formed large multimeric complexes, both *in vitro* and *in vivo*, that likely are seen as granular bodies *in vivo*.

SDS-PAGE and BiFC assays with Y10βC1 and deletion mutants of Y10βC1 indicated that multimerization was mediated by amino acids between positions 60 and 100. This sequence contains two predicted α-helices. Similarly, yeast-two-hybrid analysis indicated that self-interaction of βC1 protein encoded by Bhendi yellow vein mosaic betasatellite (BYVMB-βC1) was mediated by sequences containing two α-helices at the C-terminal half (Kumar et al., 2006). Multimerization via α-helices is a common mechanism of protein self-association. Numerous viral proteins, including the gp41 of Human immunodeficiency virus (HIV) (O'Rourke et al., 2009), the nucleocapsid protein (N) of hantaviruses (Alminaite et al., 2006) and the movement protein (MP) of Cauliflower mosaic virus (CaMV) (Stavolone et al., 2005), use this strategy.

Our results showed that Y10βC1 localized both in the cytoplasm and the nucleus and formed granular bodies in cytoplasm. Interestingly, the granular bodies in the cytoplasm formed by GFP-Y10βC1 were motile (Fig. S1). Different subcellular localization of Y10βC1 is probably associated with different functions of Y10βC1.

Multimerization is a requirement for Y10βC1 to induce begomovirus-like symptoms in *N. benthamiana*, since Y10βC1 with a C-terminal MBP tag, that is incapable of forming high order multimers, was not capable of inducing symptoms in plants when expressed using the PVX vector. The C-terminal sequences of BYVMB-βC1 were reported to interact with karyopherin α, a transport receptor involved in nuclear import (Kumar et al., 2006). A myristoylation-like motif (GMDVNE) located at the C-terminal of CLCuMB-βC1 (103 to 108aa) interacted with a ubiquitin-conjugating enzyme involved in targeting proteins for degradation by the 26S proteasome (Eini et al., 2009). So, it seems to indicate interference with a functionality associated with the C terminus of Y10βC1. But, the biological role of the multimerization is much less clear at present. Further investigations are necessary to elucidate the relationship between forming high order multimers and inducing begomovirus-like symptom.

Structural prediction indicates that the βC1 proteins encoded by distinct betasatellites likely have similar secondary and tertiary structures (data not shown). βC1 protein of ageratum yellow vein betasatellite, cotton leaf curl Multan betasatellite or Bhendi yellow vein mosaic betasatellite with GFP fused at the N-terminus also presented as granular spots in the cytoplasm and around the nucleus (Kumar et al., 2006; Saeed et al., 2007; Sharma et al., 2010), and BYVMB-βC1 self-associated via C-terminal sequence in yeast-two-hybrid assays (Kumar et al., 2006). It is likely that multimerization may be a conserved property for βC1 proteins.

Materials and methods

Plasmid constructs

The complete coding region of Y10βC1, as well as N- and C-terminal deletion mutants thereof, was amplified from pGEM-Y10β (Cui et al., 2005). The pMAL-p4X (New England Biolabs) and pCHF3 vectors (Xiong et al., 2008) were used as templates for amplifying the full coding regions of the MBP (a widely used tag for protein expression and purification) and GFP, respectively, to construct N- and C-terminal Y10βC1 fused constructs. Amplified fragments were first inserted into the plasmid vector pMD18-T (TaKaRa) and then transferred into either pET-28a (Novagen) (for protein expression in *E. coli*), the PVX vector pGR107 (Jones et al., 1999) (for symptom studies in plants), pCHF3 (for localization studies in plants) or the TMV vector pJL TRBO (Lindbo, 2007) (for native protein expression in plants). The sequences of primers used for amplification and the restriction endonuclease recognition sequences included in these for sub-cloning are listed in Table S1.

Protein expression and purification

Proteins were expressed in *E. coli* BL21 (DE3) containing pET-28a constructs at 16 °C over night at the presence of 0.1 mM IPTG. His-tagged proteins were purified in a native, non-denatured form by Ni-NTA agarose (Qiagen) as described earlier (Shao et al., 2005). Proteins were further purified by HiLoad Superdex 75 60/120 and Hitrap Heparin columns at ÄKTA purifier system (GE Healthcare) to remove any nucleic acid contamination. Finally, proteins were eluted in TN buffer (10 mM Tris-HCl, 10 mM NaCl, pH 7.5).

Cross-linking

The purified proteins were transferred from TN buffer into HN buffer (20 mM HEPES, 20 mM NaCl, pH 7.5) by gel filtration or centrifugation in Amicon Ultra-Centrifugal Filter Units (Millipore) and incubated with various concentrations of freshly prepared glutaraldehyde solution (Sigma-Aldrich) at room temperature. The reactions were quenched by the addition of 2 μl of 1 M Tris-HCl (pH 7.5). The protein samples were then boiled in SDS-denaturing buffer, separated in either 8–25% PhastGel (GE Healthcare) or 4–12% Tris-glycine gradient (Bio-Rad) polyacrylamide gels. Proteins were detected by Coomassie Blue staining and Y10βC1 was specifically detected on western blots using a monoclonal antibody raised against Y10βC1 (X. Zhou, unpublished results).

For *in vivo* cross-linking, *N. benthamiana* leaves were agroinfiltrated with pJL TRBO vector containing Y10βC1 (pTRBO:Y10βC1). Three day-post-inoculation, the intact agroinfiltrated leaves were harvested and vacuumed for 15 min in cross-linking solution containing 0.01% glutaraldehyde in 20 mM HEPES buffer (pH 8.0). After 1 h incubation at 37 °C, leaves were removed from cross-linking solution, rinsed in distilled H_2O, ground in liquid N_2, and homogenized in 20 mM Tris-HCl (pH8.0), 1% Triton X-100, 0.1 M glycine, 500 mM NaCl and 1 mM PMSF. The homogenate was incubated for 30 min at 65 °C and clarify by centrifugation. The supernatant containing target proteins was further concentrated by acetone precipitation. After air tried, pellets were resolved in 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. The proteins were fractioned on 8–16% Tris-glycine gradient gel (Bio-Rad), transferred to Hybond-C Membrane (GE Healthcare) and detected with monoclonal antibodies against Y10βC1.

Gel filtration

Gel filtration was performed with purified proteins on a pre-packed Superdex 200 column (GE Healthcare). The column was equilibrated with 10 mM Tris-HCl (pH7.4), 10 mM NaCl with a flow-rate of 1.0 mL/min at 4 °C. The elution profile was monitored by measuring A_{280nm} and A_{260nm}. The high molecular weight (HMW) gel filtration calibration kit (GE Healthcare) was used as molecular weight (MW) standards. The MW was calculated as described previously (Laurent and Killander, 1964).

Agroinoculation

Binary plasmids purified from *E. coli* cultures were introduced into *Agrobacterium tumefaciens* strain GV3101 or C58C1 by electrotransformation. Agroinoculation was performed with an overnight culture of *A. tumefaciens* carrying the appropriate plasmid as described previously (Cui et al., 2004).

BiFC assay

The full length, N- and C-terminally truncated mutants of Y10βC1 were amplified from pGEM-Y10β and ligated into the pMD18-T vector. The fragments were released by digestion with *Pac*I and *Asc*I, and inserted into the pYN1 and p2YC vectors (Hu et al., 2002) (which include codons 1 to 158 [YN domain] and 159 to 238 [YC domain] of YFP, respectively), resulting in constructs with N-terminal fusion of YN or C-terminal fusion of YC, respectively. BiFC experiments were performed as described previously (Yang et al., 2007). YFP fluorescence was observed and photographed at 2 day-post-infiltration (dpi) using a TCS SP5 confocal microscope (Leica).

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (Grant Nos. 30530520 and 30670087) and the National Key Basic Research and Development Program of China (2006CB101903).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.virol.2010.10.007.

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