A Unique Glycine-Rich Motif at the N-terminal Region of *Bamboo mosaic virus* Coat Protein Is Required for Symptom Expression

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The coat proteins (CP) of many plant viruses are multifunctional proteins. We used N-terminal sequencing and mass spectrometry/mass spectrometry analysis to identify a truncated form of the *Bamboo mosaic virus* (BaMV) CP missing the N-terminal 35 amino acids (N35). The N35 region is unique in the potexviruses by its containing a glycine-rich motif (GRM) not present in databases but highly conserved among BaMV isolates. Results from site-directed mutagenesis and deletion mutational analysis showed that loss of this region converted necrotic local lesions to chlorotic local lesions on *Chenopodium quinoa* leaves. Furthermore, this region is required for successful development of mosaic symptoms on *Nicotiana benthamiana* leaves but is dispensable for BaMV replication and cell-to-cell and long-distance movement as well as virion assembly. This unique GRM-containing region of BaMV CP may be a symptom determinant in specific hosts.

Increasing evidence shows that coat proteins (CP) of many plant viruses play multifunctional roles in almost every step of the viral infection cycle. These roles include delivery of the virus into the plant cell, disassembly of virus particles, translation of viral RNA, viral genome replication, assembly of progeny virus, virus movement between plant cells or long-distance movement in the plant, and interactions with the host plant to activate or suppress the host defenses, thus resulting in susceptibility, disease symptoms, or resistance (Bol 2005). For example, in the genus *Potexvirus*, CP is involved in virion assembly and is required for virus cell-to-cell and long-distance movement (Verchot-Lubicz et al. 2007). The CP of *Potato virus X* (PVX) is involved in genomic RNA accumulation (Chapman et al. 1992) and is responsible for induction of the Rx resistance system in potato plants (Goulden and Baulcombe 1993).

The N- and C-terminal regions of the CP of potexviruses are varied in amino acids, although the central region is highly conserved (Abouhaidar and Lai 1989). The variability of amino-acid residuals at the N terminus of potexvirus CP results in the low serological cross-reactivity among potexviruses (Koenig and Torrance 1986). Moreover, the N-terminal region may be highly sensitive to the action of plant sap proteases, and proteolytic cleavage of PVX CP may occur in situ (Koenig et al. 1970, 1978), which results in the formation of an N-terminal truncated PVX CP (Koenig et al. 1970). Such an N-terminal-truncated CP has been found in another potexvirus, *Papaya mosaic virus* (PMV), in that a large proportion of

the PMV CP of the purified virus lacked several amino acids at the N terminus (Zhang et al. 1993). However, whether this truncated CP is formed in vivo or during the processes of purification is unclear. In vitro data showed that this N-terminal region can be easily removed by mild trypsin treatment without disrupting the virion structure (Tremaine and Agrawal 1972). Further analysis showed that deletion of the N-terminal 30 amino acids of PVX CP did not substantially affect cell-to-cell movement (Chapman et al. 1992; Lico et al. 2006).

Bamboo mosaic virus (BaMV), a member of the *Potexvirus* genus, possesses a single-strand, positive-sense RNA genome of approximately 6.4 kb and contains five conserved open reading frames (ORF) (Lin et al. 1994). ORF1 encodes a 155-kDa replication-related protein with three functional domains: an N-terminal mRNA capping enzyme, a central RNA helicase, and a C-terminal RNA-dependent RNA polymerase (RdRp) (Huang et al. 2005; Li et al. 2001a and b). ORF2 through ORF4 encode movement proteins of 28, 13, and 6 kDa, respectively. These three ORF overlap in the genome and, thus, are called the triple gene block. The 25-kDa CP is encoded by ORF5. The 155-kDa protein is directly translated from genomic RNA, whereas CP is translated from the subgenomic RNA of approximately 1.0 kb. Recently, BaMV has been successfully developed as a plant-expression vector by replacing the N-terminal 35 amino acids (N35) of BaMV CP with a 37-amino acid peptide of the foot and mouth disease virus VP1. Recombinant BaMV expressing VP1 epitope induced positive immunity in swine. However, this chimeric virus causes a different symptom phenotype in *Nicotiana benthamiana* and *Chenopodium quinoa* (Yang et al. 2007).

Recently, Ozeki and associates (2009) suggested that the N-terminal region of potexvirus CP could be separated into two distinct functional domains: an N-extra (Nex) region and an N-core region. The latter region is required for cell-to-cell movement but is dispensable for virion assembly, as in *Plantago asiatica mosaic virus* (PIAMV). The former region is dispensable for both cell-to-cell movement and virion assembly. However, the exact role of the Nex region is unknown.

In the present study, we used one- and two-dimensional (1- and 2-D, respectively) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), N-terminal sequencing, and tandem mass spectrometry (MS/MS) to identify a major truncated BaMV CP lacking the N35. This region contains a unique glycine-rich motif (GRM) in the potexvirus group, which belongs to the Nex region of potexvirus CP (Ozeki et al. 2009). We found that this region is required for necrotic local lesion induction in *C. quinoa* and symptom expression in *N. benthamiana*.

RESULTS

Identification and characterization of a major truncated BaMV CP.

Initially, to elucidate whether the BaMV CP has any post-translational modifications, one-dimensional (1-D) SDS-PAGE of the purified BaMV virions and 2-D electrophoresis of BaMV CP isolated from purified BaMV virions were performed.

Freshly prepared (Fig. 1A, lane 1) and stored virions (Fig. 1A, lane 2) were used for 1-D SDS-PAGE analysis. Besides

the full-length CP (Fig. 1A, lanes 1 and 2, labeled a), a major truncated CP (Fig. 1A, lanes 1 and 2, labeled b) was always detected by SDS-PAGE, and this was confirmed by Western blot with rabbit anti-BaMV CP (Fig. 1B, lanes 1 and 2). Interestingly, a more truncated CP was detected from stored virions (Fig. 1A and B, lane 2) than from freshly prepared ones (Fig. 1A and B, lane 1). In contrast, the recombinant CP (rCP) (Fig. 1A and B, lane 3, labeled c) did not show truncated CP accumulation (Fig. 1A and B, lane 3), even after storage for a longer time (data not shown). Furthermore, both full-length and truncated

Fig. 1. One-dimensional (1-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of coat protein (CP) from purified *Bamboo mosaic virus* (BaMV) virions or infected leaves of bamboo (*Bambusa vulgaris* Schrad), as well as 2-D SDS-PAGE of CP isolated from purified BaMV virions. **A**, 1-D SDS-PAGE of BaMV virions. Freshly prepared BaMV virions (lane 1), BaMV virions stored at -70°C for 6 months (lane 2), and recombinant CP purified from *Escherichia coli* (lane 3) were used for 12% SDS-PAGE. **B**, Western blot analysis of BaMV CP. Protein samples were transferred to polyvinylidene difluoride membrane and were probed with polyclonal rabbit anti-BaMV CP at 1:5,000 and then with peroxidase-labeled goat anti-rabbit immunoglobulin G at 1:10,000. Signals were visualized with an enhanced chemiluminescence kit. **C**, CP accumulation in infected bamboo leaves was tested by 1-D SDS-PAGE (lane 1) and Western blot analysis (lane 2) as described in A and B. **D**, Proteomic assay of BaMV CP. Approximately 50 μg of CP isolated from freshly purified BaMV virions was subjected to 2-D electrophoresis. The pH range (3 to 10 NL) of the immobilized pH gradient strip (GE Healthcare) is shown on the top of the gel image. The spots numbered 1 to 12 on the gel were selected for matrix-assisted laser desorption/ionization-time of flight-based mass spectrometry or tandem mass spectrometry.

CP formed dimers (Fig. 1A, lanes 1 and 2, aa and bb), which was further confirmed by Western blot analysis (Fig. 1B, lanes 1 and 2). However, only a faint dimer was observed from rCP (Fig. 1A and B, lane 3). Such truncated CP was also observed in total proteins extracted directly from BaMV-infected leaves of different hosts, including bamboo, *C. quinoa*, and *N. benthamiana* (Fig. 1C and data not shown).

To examine whether truncation of the CP occurred at the N or C terminus, or both, we performed N-terminal sequencing and MS/MS analysis. For N-terminal sequencing, two independent experiments with different virion preparations were carried out. The direct protein sequencing showed that the residues obtained were identical to those starting from the N-terminal 36 amino acids of BaMV CP (Table 1, band b), which suggests that the truncated CP lacked the N35 region. The same peptides as those from the truncated CP were obtained by N-terminal sequencing from dimers formed by the truncated CP (Table 1, band bb), which indicated that the truncated CP was still able to form a dimer. However, no results could be obtained for the full-length CP or dimers from the full-length CP by N-terminal sequencing (Table 1, bands a and aa). Failure of N-terminal sequencing led us to suspect that the CP from virions is modified, and this modification blocks N-terminal sequencing. Therefore, we performed N-terminal sequencing of rCP. Indeed, we could sequence the N-terminal sequence of rCP in two separate experiments, and the results were as expected (Table 1, band c).

Next, to determine whether the C terminus of the truncated CP is intact, MS/MS analysis was performed. Both the monomer and dimer from the truncated CP still covered the C-terminal but not the N-terminal peptide (Table 2, bands b and bb).

Table 1. Peptides identified by N-terminal sequence

Bands	Peptides ^a
a	Failure
aa	Failure
b	AAAQPWEAIF and AAAQPWEAIFTKDDL
bb	AAAQPWEAIF and AAAQPWEAIFTKDDL
c	SGAGT and SGAGTGTGRG

^a N-terminal amino acid sequences determined by Edman degradation methodology with different *Bamboo mosaic virus* purification in two different experiments.

In contrast, both nearly intact N- and C-terminal peptides were covered on MS/MS analysis of the full-length CP and the dimer from the full-length CP (Table 2, bands a and aa). Thus, N-terminal sequencing, MS/MS analysis, and the shift of proteins on SDS-PAGE confirmed that the truncated CP lacks only the N-terminal region.

To provide more information about the BaMV CP, CP was isolated from the freshly purified virions and was separated by 2-D SDS-PAGE. Multiple forms of CP were observed on 2-D gel. The major truncated CP (Fig. 1D, spot 5) and a faint one (Fig. 1D, spot 12) with similar molecular mass but different isoelectric points (pI) were displayed. The full-length CP (Fig. 1D, spots 1 to 4 and 11) was shown as serial spots (with similar molecular mass but different pI). On 2-D gel, dimers formed from truncated CP (Fig. 1D, spots 8 to 10), and full-length CP (Fig. 1D, spot 7) appeared, even though the CP was prepared in a highly denaturing buffer system, that is, 8 M urea, 2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), and 40 mM DTT (dithiothreitol) (Fig. 1D). Interestingly, the dimers formed from truncated CP but not the full-length CP were displayed as a series of spots (Fig. 1D, spots 8 to 10). In addition, a more extensively truncated CP (Fig. 1D, spot 6) appeared. All spots were analyzed by MS/MS assay, and all spots were BaMV CP. Spots 1 to 4 and 11 were identified as full-length CP, with high protein coverage of more than 78% and both N- and C-terminal regions covered (Table 2, spots 1 to 4 and 11). Spots 5, 6, and 12 were identified as the truncated CP lacking the N-terminal region (spots 5 and 12) or both N- and C-terminal regions (spot 6) (Table 2). In general, spots corresponding to the dimers formed by truncated CP had low protein coverage, less than 30% (Table 2, spots 8 to 10), whereas the dimer formed from the full-length CP had high protein coverage, with both N- and C-terminal regions covered (Table 2, spot 7). MS/MS spectra revealed no modifications at the N terminus of BaMV CP, although spot 1 was identified to be a phosphorylated CP at the C terminus (data not shown).

The N35 region contains a unique GRM.

The N35 region contains a GRM (19 glycines out of the 35 amino acids) that could form a coiled-coil structure as predicted by Pôle BioInformatique Lyonnaise network program sequence analysis software. Phylogenetic analysis of CP of the potexvirus group revealed that although BaMV is closely

Table 2. Tandem mass spectrometry identification of the *Bamboo mosaic virus* (BaMV) coat protein (CP) from one- and two-dimensional gels

Bands and spots	GI number ^a	Protein name	Protein covered (%)	Amino-acid sequence matched to BaMV CP
BaMV CP ^b	2407623	¹ MSGAGTGTGRGTGTGVGGTGGTGGTGGGGTGRGQAAAQPWEAIFTKDDLAAIEPKPASANVPNTKQWI GIQAGLIKAGATDANFMKVLGLSLAEAFDRGSSEATTWDGITEGVVEHRAAANAIEKNCPIHKVITYYLAKP TFAIRQSKNLPPANFAKKNVPSQYKWCADFADGLYDPTCLASELPYDAPSEIDRMAYATFKTIQIKIANDQ KGFNLNYPNVTQARLPN APLPALPEPTSD ²⁴²		
a	2407623	BaMV CP	53.31	G ¹¹ -R ³² , D ⁴⁸ -K ⁶⁶ , A ⁷⁸ -R ⁹⁹ , E ¹²⁵ -R ¹⁴⁵ , N ¹⁴⁹ -K ¹⁵⁷ , M ¹⁹⁶ -K ²⁰² , G ²¹⁴ -D ²⁴²
aa	2407623	BaMV CP	38.84	G ¹¹ -R ³² , D ⁴⁸ -K ⁶⁶ , G ¹⁰⁰ -R ¹¹⁷ , I ²⁰⁸ -D ²⁴²
b	2407623	BaMV CP	29.75	D ⁴⁸ -K ⁶⁶ , G ¹⁰⁰ -R ¹¹⁷ , I ²⁰⁸ -D ²⁴²
bb	2407623	BaMV CP	14.05	D ⁴⁸ -K ⁶⁶ , I ²²⁸ -D ²⁴²
1	2407623	BaMV CP	86.36	M ¹ -R ¹⁴⁵ , N ¹⁴⁹ -K ¹⁶⁵ , M ¹⁹⁶ -D ²⁴²
2	2407623	BaMV CP	80.17	M ¹ -R ¹¹⁷ , V ¹³³ -R ¹⁴⁵ , N ¹⁴⁹ -K ¹⁶⁵ , M ¹⁹⁶ -D ²⁴²
3	2407623	BaMV CP	78.93	M ¹ -R ¹⁴⁵ , E ¹²⁵ -R ¹⁴⁵ , N ¹⁴⁹ -K ¹⁶⁵ , M ¹⁹⁶ -K ²⁰² , G ²¹⁴ -D ²⁴²
4	2407623	BaMV CP	93.39	M ¹ -R ¹⁴⁵ , E ¹²⁵ -R ¹⁴⁵ , N ¹⁴⁹ -K ²⁰⁷ , G ²¹⁴ -D ²⁴²
5	2407623	BaMV CP	49.17	D ⁴⁸ -K ⁶⁶ , V ⁸⁸ -R ⁹⁹ , E ¹²⁵ -K ¹³² , P ¹⁴⁰ -R ¹⁴⁵ , K ¹⁵⁸ -K ²⁰² , G ²¹⁴ -D ²⁴²
6	2407623	BaMV CP	10.74	V ⁸⁸ -R ⁹⁹ , G ²¹⁴ -R ²²⁷
7	2407623	BaMV CP	71.90	M ¹ -R ¹¹⁷ , V ¹³³ -R ¹⁴⁵ , K ¹⁵⁸ -K ¹⁶⁵ , M ¹⁹⁶ -K ²⁰² , G ²¹⁴ -D ²⁴²
8	2407623	BaMV CP	12.81	P ⁵⁷ -K ⁶⁶ , M ¹⁹⁶ -K ²⁰² , G ²¹⁴ -R ²²⁷
9	2407623	BaMV CP	13.22	P ⁵⁷ -K ⁶⁶ , A ⁷⁸ -R ⁹⁹
10	2407623	BaMV CP	11.98	D ⁴⁸ -K ⁶⁶ , A ⁷⁸ -K ⁸⁷
11	2407623	BaMV CP	78.93	M ¹ -R ¹¹⁷ , E ¹²⁵ -R ¹⁴⁵ , N ¹⁴⁹ -K ¹⁶⁵ , M ¹⁹⁶ -K ²⁰⁷ , G ²¹⁴ -D ²⁴²
12	2407623	BaMV CP	34.30	D ⁴⁸ -K ⁶⁶ , V ⁸⁸ -R ⁹⁹ , E ¹²⁵ -K ¹³² , K ¹⁵⁸ -K ¹⁶⁵ , M ¹⁹⁶ -K ²⁰² , G ²¹⁴ -D ²⁴²

^a Assigned by the National Center for Biotechnology Information.

^b BaMV CP amino acid sequence deduced from nucleotide sequence of the BaMV CP open reading frame.

A	CVX (AY241392)	--MSTTGVSQ	SQSSGPRSTP	QSGPFQTLSS	SQLAALS----
	ZVX (YP_054406)	-MSN.A....	..T..V.APA	TPS.....TA	A.....-----
	PMV (NP_044334)	---.KSSMST	PNIAF.AI.Q	EQMSSIKVDP	TSNLLP.----
	NdMV (AY800279)	----MALTAA	PTTDALAAMT	FQVSSPSVPT	ASELNTITQ--
	PlMV (NC_003849)	----MALNTA	PTADALAAMA	FPVSSPSVPT	A.ELDTITS--
	CsCMV (NP_042699)	-MATP.STTP	TTATITQAAT	TPLSALSTAP	TDEELS-----
	HRSV (YP224089)	-MTT..TTNP	IPPQAGT...	SAAFAPLSQE	VLETLS-----
	CYMV (P16486)	-----MTDT	KKTLEFSAP.D	EQLDTL..TI	ESNLVP.ISEL
	CyBMV (ABB20690)	-MGEP----T	PAPAATY.AA	DPTSAPR.AD	LAAIKY.PVT-
	PAMV (NP_619750)	-MVDSKKTET	P.VIDASKKT	E.SKTSHAGR	V.FLSA-----
	NMV (NP_040782)	-MATPSTQTT	DPKPANADLS	DPNRAPS.ED	LKKIKY-----
	PpMV (AAV51618)	-MADN.P.AA	TSG.P.TAQD	AGAKAPADF.	NPNT.P-----
	WClMV (DQ784572)	-MAT..ATTP	PSLTDI.ALK	YISSTVSVA.	PAEIEA-----
	NVX (YP_446996)	MANTSASAAL	PAQ.KTDDMT	APPDNKD.EA	L.YIP-----
	PVX (AAV27212)	-MTTPANTTQ	ATG.TTST.T	KTAGATPATT	.G.FTI-----
	HsLV (AAZ15110)	-MATKVADNP	.DPNVGEPKA	TKPGDNVDP	AAADLF-----
	LVX (CAA33396)	-----MTTFV	PDAKTWAD.A	YTAQSESVAT	AEELQSIATL-
	SMYEAV (NP_62046)	-MGDQPRPPV	PPAP.SNPL.	MGSTPPV.PG	RTPNPN-----
	FMV (NC_001483)	-MATQNADVT	DATDYKKPPA	ETEQAALTIQ	PRSNKA-----
	BaMV-S (AAB70566)	-MSGAGTGTG	RGTGTGVGGT	GTGGTGGGG	TGRGQQ-----
B	BaMV-S	MSGAGTGTGR	GTGTGVGGTG	GTGGTGGGGT	GRGQQ
	BaMV-O	...T.....			
	BaMV-V	...T.....	...A....	D...	
	BB2CP	...T.....	...A.A....	S....	
	BB13CP1	...T.....	...A....		
	BB30CP	...T.N....	...T.N.	D...P....	
	BB34CP	...T.....			
	BO3CP1	...T.....	...A....		
	BO14CP1	...T.S....	.A..A....		
	BO18CP2	...N.....	...A....		
	BO23CP1	...T.....	...A....		
	BO31CP	...T.....	...A....		
	BV11CP1	...T.....	...G....		
	DL15-1-CP1	...T.....	...G....	V....	
	DL25CP	...T.....	...A....		
	S5-CP5	...T.....	...A....		
	BB5CP	...T.....	...T....	...S.D....	
	BB14CP1	...T.....	...A....		
	BB31CP	...T.....		A.....	
	BB35CP	...T.....		A.....	
	BB48CP1	...T.....	...A....		
	BO11CP2	...T..A..	...A....		
	BO15CP1	...T.....			
	BO19CP1	...T.....	...A....		
	BO29CP	...T.....	...A....		
	BO32CP1	...T.....	.N..A....		
	BV17CP1	...T.....	...A....		
	DL22	...T....G	...T....	...S...A	
	S1-CP1	...T.....	...A....		
	BB12CP1	...T....V	...T....		
	BB26CP1	...T.....	...T....	...S....	
	BB42CP	...T.....			
	BL	...T.....	...A....	D....	
	BO13CP	...T.....	...A....	...S....	
	BO17CP1	...T.....	...G....	...S....	
	BO22CP2	...T.....	...G....		
	BO30CP	...T.....	...A....		
	BO34CP	...T.....	...A....		
	DL12CP1	...T.....	...G....		
	DL24-CP	...E.....	...T....	...R....	
	S2-CP2	...T.....	...A....		

Fig. 2. Multiple alignments of *Bamboo mosaic virus* (BaMV) coat protein (CP) N-terminal 35 amino acids. **A**, Multiple alignments of the N-terminal 35 amino acids of CP from the potexvirus group. MEGA 4 software was used to align the deduced polypeptide sequence. Identical residues are indicated by a dot (•), and gaps are indicated by a dash (–). The glycine-rich motif of BaMV CP is underlined. **B**, Comparison of the N-terminal amino acids of CP among BaMV isolates. Residues identical to those of the BaMV-S isolate (AF018156) are indicated by a dot (•).

related to *Foxtail mosaic virus* (Yang et al. 1997), this GRM is unique to BaMV among potexvirus groups (Fig. 2A). No similar sequence has been found in the database. Furthermore, this GRM is highly conserved in BaMV isolates collected around the world (Fig. 2B).

Mutational analysis of the N35 region in *N. benthamiana* protoplasts.

To determine the biological function of the N35 region of CP in planta, various mutations were introduced into the BaMV

CP region by deletion or site-directed mutagenesis (Fig. 3A). For CPstop, the start codon AUG was altered to stop codon UGA and disrupt CP translation. However, two other in-frame AUG codons in the CP ORF (Fig. 3B) could produce peptides with 157 and 47 amino acids in length, respectively (if these AUG sequences could function as start translation codons). The nucleotides at position -3 of these two putative translation initiation codons are pyrimidines U and C instead of a purine A or G, respectively (Fig. 3B), which is not an optimal start codon context for protein translation (Kozak 1986, 2002). For

Fig. 3. Mutational analysis of the function of the N-terminal 35 amino acids of *Bamboo mosaic virus* (BaMV) coat protein (CP). **A**, Schematic representation of the structure of the wild type (wt) BaMV CP and derivatives. The genome of BaMV-S isolate (AF018156) contains five open reading frames. Nucleotide sequences corresponding to the CP start (⁵⁴⁹⁵aagATG⁵⁵⁰⁰) and stop (⁶²²⁶TAA⁶²²⁸) codon regions are indicated. The N-terminal 39 amino acids encoded by the wt CP gene are shown. The arrow indicates where the truncated CP was formed. The solid lines of the mutants indicate that the N-terminal 35 amino acids were removed but the nucleotides were retained, and the line of dashes indicates that the nucleotides were removed. Asterisk indicates the stop codon. **B**, Nucleotide sequence in CP start regions of wt and derivatives of BaMV. **C**, Northern blot analysis of BaMV RNA accumulation in infected *Nicotiana benthamiana* protoplasts. *N. benthamiana* protoplasts were inoculated with in vitro transcripts from wt and mutants of BaMV at 24 h postinoculation (hpi). Total RNA extracted from approximately 1×10^4 protoplasts was loaded in each lane. The accumulation of BaMV RNA was measured using a BaMV-specific probe (Lin et al. 1996). Equal loading is shown as ethidium bromide staining. **D** and **E**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis of CP accumulation in *N. benthamiana* protoplasts at 24 hpi. **D**, Western blot analysis of BaMV CP accumulation measured from approximately 1×10^4 infected protoplasts by rabbit anti-BaMV CP. Purified BaMV virions (about 0.5 μ g) were used as a positive control (lane 8). The thin arrow indicates the full-length CP, and the bold arrow indicates the truncated CP. **E**, Equal loading was shown as Coomassie Brilliant Blue G-250 staining.

Δ CPN35, nucleotides (from 5,501 to 5,602) encoding the second Ser to the 35th Gln were deleted to produce a truncated CP lacking the N35 region. CPQ³⁵/M was created on the basis of the CPstop and contains the additional substitution of the Gln codon CAA at amino acid 35 with the start codon AUG, to truncate the amino acid sequence from the second Ser to the 35th Gln but retain the nucleotides. CPQ³⁵/M_{AAA} was created on the basis of CPQ³⁵/M and contains an additional nucleotide substitution of pyrimidine C at position -3 of the created start codon AUG with a purine A, to increase CP translation efficiency (Fig. 3B). For CPA³⁷A³⁸/PP, the N-terminal 37th and 38th Ala of wild-type (wt) CP were replaced by two prolines to prevent the truncation of the N35 region. Transcripts were synthesized *in vitro* from the wt and mutants and were inoculated into *N. benthamiana* protoplasts. Total RNA and protein was extracted and analyzed at 24 h postinfection (hpi).

Northern blotting showed that CPstop and Δ CPN35 accumulated to near-wt levels, whereas CPQ³⁵/M, CPQ³⁵/M_{AAA}, and CPA³⁷A³⁸/PP accumulated to a slightly higher level in the infected protoplasts (Fig. 3C). These results suggest that all constructed mutants can replicate their genomic RNA at a single-cell level and indicate that the nucleotide sequence of the N35 region does not function as a *cis*-acting element mediating BaMV accumulation.

As expected, Western blot analysis with a specific antibody against BaMV CP revealed no CP in protoplasts infected with CPstop (Fig. 3D, lane 2), which suggests that either the other two AUG codons did not function as translation initiation codons or the translation efficiency is very low and the amount of protein was too low to be detected. Alternatively, the shorter peptide of CP is not stable. As shown in Figure 3D, protoplasts inoculated with Δ CPN35 (lane 3) accumulated an amount of

CP nearly similar to that of the wt (lane 1) and CPA³⁷A³⁸/PP (lane 6), based on the equal loading of each sample (Fig. 3E). Unexpectedly, no CP was detectable in protoplasts infected with CPQ³⁵/M at 24 hpi (lane 4). This finding may be due to low translation efficiency because the start codon context caaAUGgcu of mutant CPQ³⁵/M (Fig. 3B) with the -3 position a pyrimidine C rather than a purine A or G is not a strong translation context according to the Kozak rule (Kozak 1986, 2002). To test this possibility, CPQ³⁵/M_{AAA} was generated from CPQ³⁵/M by changing the pyrimidine C at the -3 position of the start codon context to a purine A, creating a strong start codon context (Fig. 3B). Still no CP was detectable in protoplasts infected with CPQ³⁵/M_{AAA} (Fig. 3D, lane 5). These results suggest that, in addition to the start codon context, the 5' untranslated region (UTR) length or structure of the CP mutant may affect CP translation.

Losing the N35 region causes chlorotic local lesions on *C. quinoa* plants.

Although all of the tested mutants could replicate well in protoplasts independent of CP mutation (Fig. 3), the relationship between the biological relevance of the N35 region of BaMV CP in infectivity and symptom development remained to be determined in plants. As shown in Figure 4A, substantial necrotic local lesions were induced on *C. quinoa* leaves inoculated with wt BaMV at 8 days postinoculation (dpi), whereas blocking CP translation in CPstop resulted in no visible local lesions on *C. quinoa* leaves as were seen on mock-inoculated leaves. Lack of the N35 region in Δ CPN35 and CPQ³⁵/M_{AAA} led to chlorotic rather than necrotic local lesions on *C. quinoa* leaves. At 8 dpi, only a few local lesions were induced on *C. quinoa* leaves by CPQ³⁵/M, which produced a lower level of CP (Fig. 4B, lane 3),

Fig. 4. Infectivity of wild type (Wt) and mutants of *Bamboo mosaic virus* (BaMV) on *Chenopodium quinoa* plants. **A**, Representative examples of symptoms induced by BaMV and BaMV mutants on inoculated leaves of *C. quinoa*. Photographs were taken at 8 days postinoculation. **B** and **C**, Western blot analysis of coat protein (CP) accumulation. **B**, Western blot. Purified BaMV virions (about 0.5 μ g) were used as a positive control. **C**, Equally loading is shown as Coomassie Brilliant Blue G-250 staining. **D**, Reverse transcription-polymerase chain reaction amplification of the CP gene.

and the local lesion number did not increase with increased concentration of inoculum (from 0.1 to 1 µg per leaf). In contrast, CPQ³⁵/M_{AAA} produced more CP product (Fig. 4B, lane 4) and induced more local lesion numbers. Surprisingly, inoculation of CPA³⁷A³⁸/PP resulted in more severe local lesions than those of the wt and other mutants (Fig. 4A).

Western blot analysis revealed that *C. quinoa* leaves inoculated with wt BaMV accumulated full-length and truncated CP (Fig. 4B, lane 1). No CP was detected on *C. quinoa* leaves inoculated with CPstop (lane 6) as was found in *N. benthamiana* protoplasts, which indicates that CPstop is, indeed, a CP null mutant. *C. quinoa* leaves inoculated with ΔCPN35 accumulated substantial CP (lane 2). At 8 dpi, faint CP was detected on *C. quinoa* leaves inoculated with CPQ³⁵/M (lane 3), which suggests that its CP ORF is translatable but the translation efficiency is low. A significant amount of CP was produced on leaves inoculated with CPQ³⁵/M_{AAA}, with strong translation context (lane 4). *C. quinoa* leaves inoculated with CPA³⁷A³⁸/PP always showed a much higher CP level than that for the wt and other mutants (lane 5), which corresponded well with local lesion development.

To detect the BaMV RNA accumulation, total RNA was extracted from *C. quinoa* leaves inoculated with CPstop, CPQ³⁵/M, and CPQ³⁵/M_{AAA} at 8 dpi for reverse transcription-polymerase chain reaction (RT-PCR) analysis. A trace amount of genomic RNA was detected on *C. quinoa* leaves inoculated with CPstop (Fig. 4D, lane 1), which indicates that CPstop could replicate in single cells but could not move cell to cell. In agreement with the local lesion development, substantial genomic RNA was detected on *C. quinoa* leaves inoculated with CPQ³⁵/M (Fig. 4D, lane 2) and CPQ³⁵/M_{AAA} (Fig. 4D, lane 3).

To further examine whether these mutants are stable, PCR products were cloned into plasmid pGEMT-easy vector with TA cloning. Ten colonies for each mutant were chosen for sequencing. Sequencing results showed that the mutated sites

were still maintained (data not shown), which suggests that the symptoms observed are indeed induced by the generated mutations.

The N35 region is required for symptom formation on *N. benthamiana* plants but is dispensable for BaMV long-distance movement.

The biological significance of the N35 region of BaMV CP in infectivity and symptom development was further examined in the systemic host *N. benthamiana*. No visible symptoms could be observed on mock- and CPstop-inoculated *N. benthamiana* leaves (Fig. 5). Unexpectedly, no visible symptoms other than slight yellowing on some leaves were induced on leaves inoculated with ΔCPN35, CPQ³⁵/M, and CPQ³⁵/M_{AAA}. Again, severe mosaic symptoms and yellowing were induced on leaves inoculated with CPA³⁷A³⁸/PP.

Northern blot analysis revealed that the wt and all mutants except CPstop induced BaMV genomic RNA accumulation on both inoculated and systemic *N. benthamiana* leaves (Fig. 6A), which indicates that all mutants except CPstop could mediate the cell-to-cell and long-distance movement of their RNA. Of note, much less genomic RNA was detected in leaves inoculated with CPQ³⁵/M and CPQ³⁵/M_{AAA} (Fig. 6A, lanes 3 and 4) than in leaves inoculated with the wt BaMV and other mutants, except CPstop. This finding could be due to the relatively low CP level of the constructs, which resulted in less cell-to-cell movement than replication activity (Fig. 3). In agreement with the severity of symptoms, the highest level of genomic RNA was detected on both inoculated and systemic leaves inoculated with CPA³⁷A³⁸/PP (Fig. 6A, lanes 5 and 12).

Western blot analysis revealed CP accumulated on both inoculated and systemic leaves inoculated with both wt and mutants, with the exception of CPstop (Fig. 6B). CP level was associated with genomic RNA level; that is, the CP level was the

Fig. 5. Representative examples of symptoms induced by *Bamboo mosaic virus* (BaMV) and BaMV mutants on systemic leaves of *Nicotiana benthamiana*. Photographs were taken at 20 days postinoculation.

highest and lowest on both inoculated and systemic leaves inoculated with CPA³⁷A³⁸/PP (Fig. 6B, lanes 5 and 12) and CPQ³⁵/M (Fig. 6B, lanes 3 and 10), respectively, according to the equal loading (Fig. 6C). The amount of CP was restored to a

Table 3. Summary of symptom expression and formation of particles of wild type (wt) *Bamboo mosaic virus* (BaMV) and derivatives

Transcripts	<i>Chenopodium quinoa</i>		<i>Nicotiana benthamiana</i>	
	Local lesions ^a	Virions ^b	Symptoms ^c	Virions ^b
wt	>100	+	Mosaic	+
CPstop	–	–	–	–
ΔCPN35	>100	+	Light yellowing	+
CPQ ³⁵ /M	<5	–	–	+
CPQ ³⁵ /M ^{AAA}	<100	+	Mild mosaic	+
CPA ³⁷ A ³⁸ /PP	>100	+	Mosaic, yellowing	+

^a The estimated number of local lesions on inoculated leaves at 8 days postinoculation. – indicates no visible local lesion.

^b Formation of virions assayed by electron microscopy or serologically specific electron microscopy. + indicates virus particles were observed; – indicates no virus particles observed.

^c Typical symptoms induced on *Nicotiana benthamiana* by BaMV. – indicates no visible symptoms.

significant level on both inoculated and systemic leaves inoculated with CPQ³⁵/M^{AAA} (Fig. 6B, lanes 4 and 11), although less than that with ΔCPN35 (Fig. 6B, lanes 2 and 9). In addition, truncated CP was detected on systemic leaves inoculated with wt BaMV (Fig. 6B, lane 8) and CPA³⁷A³⁸/PP (Fig. 6B, lane 12).

N35 region is dispensable for BaMV virion formation.

To test virion formation, crude sap of inoculated *C. quinoa* leaves and systemic *N. benthamiana* leaves was examined by negative staining or serologically specific electron microscopy (SSEM). As summarized in Table 3, we observed virions on *C. quinoa* leaves inoculated with wt and all mutants except CPstop and CPQ³⁵/M at 8 dpi. Similarly, virions were detected on the systemic *N. benthamiana* leaves inoculated with wt and all mutants except CPstop, at 20 dpi, which suggests that the N35 region is dispensable for virion assembly.

DISCUSSION

Previously, an N terminal-truncated CP of PVX and PMV was found from purified virions (Koenig et al. 1970, 1978; Zhang et al. 1993). However, the mechanism underlying the for-

Fig. 6. Infectivity assays of wild type (wt) and mutants of *Bamboo mosaic virus* (BaMV) in *Nicotiana benthamiana* leaves. **A**, Northern blot analysis of BaMV RNA accumulation in *N. benthamiana* leaves inoculated with in vitro transcripts of wt and mutants of BaMV. Accumulation of BaMV RNA was assayed in inoculated leaves at 8 days postinoculation (dpi) and systemic leaves at 20 dpi. **B** and **C**, Western blot analysis of coat protein (CP) accumulation in inoculated and systemic leaves of *N. benthamiana*. **B**, Western blot. The thin arrow indicates the full-length CP, and the bold arrow indicates the truncated CP. **C**, Equal loading was shown as Coomassie brilliant blue G-250 staining.

mation of this truncated CP remains unclear, and its biological importance is unknown. In this work, we found a major truncated CP from both purified BaMV virions (Fig. 1A and B) and total proteins from different infected hosts (Figs. 1C, 4 and 6). Several lines of evidence suggest that the truncated BaMV CP from virions is not generated during the purification, and its formation is likely related to CP post-translational modifications. First, rCP did not have a tendency to form a truncated CP (Fig. 1A and B, lane 3), possibly because of lack of modifications. Second, multiple forms of CP were found on 2-D SDS-PAGE and were identified by MS/MS analysis (Fig. 1D; Table 2), which demonstrates that the BaMV CP may be modified. Although MS spectra did not reveal any modifications at the N terminus, some units of CP are phosphorylated at the C terminus (data not shown). In addition, the N-terminal sequence of the full-length CP could not be obtained by N-terminal sequencing, which further suggests that the N terminus may be blocked by unknown modification (Table 1). Motif scanning predicated five myristyl sites in this N35 region (MyHits Motif scan website). These modifications may result in substantial changes in physicochemical properties of the BaMV CP N terminus, leading to the easy truncation of the N35 region in a physical or chemical manner. Finally, although the same method was adopted in this study to extract the total protein from different materials, truncated CP was undetectable in BaMV-infected *N. benthamiana* protoplasts (Fig. 3D) and in inoculated *N. benthamiana* leaves (Fig. 6B), which suggests that the formation of the truncated CP is not due to the extraction process but is more related to one or both the specific tissues or hosts as well as the infection cycle. In addition, one more extensively truncated CP was observed in bamboo (Fig. 1C) and *C. quinoa* leaves (Fig. 4B) as well as purified virions (Fig. 1D, spot 6), which reflects the dynamic truncation of CP in vivo.

The N35 region of BaMV CP contains a conserved GRM (Fig. 2) with no significant homology with any known sequences to date. According to the recent proposal by Ozeki and associates (2009), the N35 region of BaMV CP belongs to the Nex region, with functions to be defined in potexvirus. Predicted results showed that this GRM-containing region could form a coiled-coil structure, which could mediate protein-protein interaction. Consistent with previous studies of other potexviruses (Chapman et al. 1992; Lico et al. 2006; Ozeki et al. 2009), the N35 region is not required for BaMV replication (Figs. 3C and 6A), virus assembly (Table 3), cell-to-cell movement (Figs. 4 and 6), or long-distance movement (Figs. 5 and 6). Furthermore, mutational analysis confirmed that the nucleotide sequence of the N35 region of BaMV CP does not function as a *cis*-regulatory element for BaMV replication (Fig. 3C). Because potexvirus CP has distinct functions in cell-to-cell and systemic movement (Lico et al. 2006), the potexvirus is generally believed to move from cell to cell via the interaction of host proteins and the ribonucleoprotein complex (RNP), which is composed of viral RNA, CP, and TGBp1 (Verchot-Lubicz et al. 2007). Because the mutant Δ CPN35 could still move from cell to cell (Figs. 4, 5, and 6), it is suggested that the N35 region does not affect RNP formation or the interaction of host proteins and RNP. In contrast, the long-distance movement of potexvirus is mediated by assembled virus particles through phloem (Verchot-Lubicz et al. 2007). Our findings that deletion of the N35 region did not block virion formation and systemic movement also support the idea that long-distance movement of BaMV mutants was mediated by assembled virus particles through phloem. This result also indicates that the mutated virus particles could still interact with host proteins to load into and unload its virus from phloem. The truncated CP may still exert the two main functions, cell-to-cell movement and virus assembly, because of the high stability of dimers both from full-length and truncated BaMV CP, even

under highly denaturing conditions (Fig. 1). Previous studies suggested that dimer formation of CP may be a prerequisite for its function (Krol et al. 1999; Tenllado and Bol 2000). The C-terminal mutations rather than other N-terminal mutations of *Alfalfa mosaic virus* CP can affect dimer formation, thus leading to no virion assembly (Choi and Loesch-Fries 1999). Collectively, the data suggest that the C terminus of BaMV CP may be involved in dimer formation.

One common response of plants to virus invasion is developmental defects resulting in symptom expression. Symptoms are the result of perturbation and interference in the regulation of genes involved in plant growth and development (Revers et al. 1999). The role of viral CP in symptom expression has been reported in several viruses (Banerjee et al. 1995; Bol 2005; Culver and Dawson 1989; Knorr and Dawson 1988; Neelman et al. 1991; Shintaku and Palukaitis 1992). Some CP themselves act as an elicitor inducing a hypersensitive reaction (HR) in specific hosts. For example, *Tobacco mosaic virus* CP 25 is an elicitor molecule responsible for the induction of HR in *Nicotiana sylvestris* (Culver and Dawson 1989). The CP of tobamoviruses, including *Paprika mild mottle virus* and *Pepper mild mottle virus*, are the elicitor of the capsicum L(2) and L(4) gene-mediated HR (Gilardi et al. 2004). In the potexviruses, PVX CP has been reported to be a strain-specific elicitor of Rx1-mediated virus resistance in potato (Bendahmane et al. 1995). In some cases, one amino-acid change of CP results in different symptom expression. For instance, the amino-acid residue at position 151 of the CP of *Cowpea chlorotic mottle virus* (CCMV) determines the symptom phenotype of strains CCMV-T and CCMV-M on cowpea (de Assis Filho et al. 2002). Similarly, a single amino-acid substitution in the CP of *Cucumber mosaic virus* induces chlorosis in tobacco (Shintaku and Palukaitis 1992). However, few reports of the N-terminal region of potexvirus CP, especially the Nex, in symptom expression are available. Our studies clearly support that the Nex of BaMV CP is a symptom determinant in *C. quinoa* and *N. benthamiana*. How the N35 region interacts with the hosts leading to the misregulation of genes involved in the symptom expression should be explored.

Because the N35 region of BaMV plays an important role in symptom expression and is easy to be truncated in vivo, we attempted to explore the consequence of preventing the N35 region truncation by generating CPA³⁷A³⁸/PP. Presumably, the truncated CP was formed by an unknown proteinase, as was previously suggested (Koenig et al. 1978). The putative enzyme that could cleave the peptide bond between amino acids Gln and Ala at positions 35 and 36 of BaMV CP was predicted to be thermolysin (EC 3.4.24.27) by the ExPaSy PeptideCutter program. The enzyme is a thermostable neutral metalloproteinase produced by the gram-positive bacteria *Bacillus thermoproteolyticus* (Endo 1962) and specifically catalyzes the hydrolysis of peptide bonds containing hydrophobic amino acids. The cleavage should be blocked in the mutant CPA³⁷A³⁸/PP with the 37th and 38th Ala of CP replaced by Pro (Keil 1992). Although such mutation did not totally block the formation of truncated CP (Figs. 4B, lane 5 and 6B, lane 12), the mutant showed higher accumulation of full-length CP and stronger symptom development than did the wt BaMV, which further confirms our conclusion that the N35 containing the conserved GRM of BaMV CP is required for symptom formation.

MATERIALS AND METHODS

Constructs of BaMV cDNA mutants for infectivity assay.

Plasmid pBS2-8, harboring a full-length cDNA of BaMV isolate S (BaMV-S, EMBL/GenBank accession number AF018156) with a T7 promoter fused upstream of the 5' UTR

and a poly (A) tail downstream of the 3' UTR, was constructed by inserting the full-length cDNA of BaMV-S into plasmid pUC119 with restriction enzymes *Xba*I and *Sac*I (Lin et al. 2004). Plasmid pBS2-8 hereafter is called wt BaMV cDNA and, unless otherwise indicated, was used as the template to generate the deleted or site-directed cDNA mutants by standard molecular methods (Sambrook et al. 2000).

To create the cDNA mutant with the CP translation disruption, we generated a cDNA mutant, CPstop. The first fragment was generated from pBS2-8 with primers CP1f (5'-CACTATGCATCATTCTGTCCATAC-3'), corresponding to nucleotides 5,314 to 5,337 of BaMV-S (this was also applied to other primers unless indicated), and CPstopr (5'-AGCTCCAGATcctttagtg-3'; the position of start codon AUG of the CP ORF is underlined and was replaced by the stop codon UGA, denoted as lowercase letters), corresponding to nucleotides 5,490 to 5,509. Similarly, the second fragment was synthesized with primers CPstopf (5'-CACTAAAGtgatctggagct-3'), corresponding to nucleotides 5,490 to 5,509, and CP4r (5'-CCAGTCACGACGTTGTAAAA-3'), corresponding to nucleotides 302 to 322 of pUC119. Next, overlapping PCR was performed to link the first and second fragments with the primer pair CP1f and CP4r, with the mixture of the first and second fragments used as the PCR template. After mutagenesis, the final PCR product was digested with *Nsi*I and *Sac*I and was subcloned into pBS2-8 digested with the same set of restriction enzymes to replace the corresponding fragment of wt cDNA. The same strategy was applied to construct the other cDNA mutants with their respective primers.

For ΔCPN35, the primer pair ΔCPN35r (5'-TCCCAGGGCTGGGCTGCAGCCATCTTTAGTGTTTAATTTA-3'), corresponding to nucleotides 5,481 to 5,500 and 5,603 to 5,622, was used instead of CPstopr, and ΔCPN35f (5'-TAAATTAAACACTAAAGATGGCTGCAGCCAGCCCTGGGA-3') complementary to ΔCPN35r was used instead of CPstopf.

CPQ³⁵/M was created, with CPstop instead of pBS2-8 used as the PCR template with the primers CPQ³⁵/Mr (5'-GCTGCA GCcatTTGCCCTCTA-3'; the position of the created codon AUG is underlined, and lowercase letters indicate the mutated nucleotides), corresponding to nucleotides 5,590 to 5,610, and CPQ³⁵/Mf (5'-TAGAGGGCAaatgctgcagc-3'), complementary to CPQ³⁵/Mr.

CPQ³⁵/M_{AAA} was constructed with CPQ³⁵/M used as the PCR template with the primers CPQ³⁵/M_{AAA}r (5'-GCTGCAGCcatTTCCCTCTA-3'; lowercase letters indicate the mutated nucleotides), corresponding to nucleotides 5,590 to 5,610, and CPQ³⁵/M_{AAA}f (5'-TAGAGGGGAaatgctgcagc-3'), complementary to CPQ³⁵/M_{AAA}r.

CPA³⁷A³⁸/PP was constructed with pBS2-8 used as the PCR template with the primers CPA³⁷A³⁸/PPr (5'-AGGGCTGtggtgagcttggctccctcta-3'; lowercase letters indicate the mutated nucleotides), corresponding to nucleotides 5,590 to 5,618, and CPA³⁷A³⁸/PPf (5'-TAGAGGGCAACAAGCTccaccacagccct-3'). All the final constructs were confirmed by DNA sequencing.

Construct used for overexpressing recombinant CP in *Escherichia coli*.

The CP ORF was generated from plasmid pBS2-8 by PCR with the primers CPndel (5'-CTAcatATgTCTggAgCTggAACgggAA-3'; the underlined indicates the *Nde*I site) and CPxhoI (5'-ATgCTCgAgTTAgTCTgATgTTggTTCgggA-3'; the underlined indicates the *Xho*I site). The PCR product was digested with *Nde*I and *Xho*I and cloned into plasmid pET21b (Novagen, Madison, WI, U.S.A.) restricted with the same set of enzymes. The final plasmid was confirmed by sequencing and named pET21CP.

In vitro capped RNA synthesis and inoculation.

In vitro capped RNA synthesis was performed mainly as described (Lin et al. 2004). Briefly, plasmids were linearized at the 3' end of the poly (A) tail with the restriction enzyme *Sac*I and were purified twice with phenol/chloroform/isoamyl alcohol (25:24:1). Capped RNA was synthesized with a linearized plasmid directed by a T7 promoter in the presence of m⁷GpppG.

For whole-plant inoculation, true leaves of one-month-old *C. quinoa* or *N. benthamiana* plants were inoculated with wt or various mutant transcripts with about 0.1 μg of transcript per leaf. For inoculating protoplasts, approximately 2 × 10⁵ protoplasts isolated from *N. benthamiana* suspension cells were transfected with about 1 μg of transcripts by electroporation as described previously (Lin et al. 1992).

RT-PCR and northern blot analysis.

Total RNA was extracted from infected protoplasts, inoculated leaves, and systemic leaves. For RT-PCR, 1 μg of DNA-free total RNA was used to synthesize cDNA by the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, U.S.A.) according to the procedure provided. cDNA (diluted 10-fold) was used as a PCR template with the primers CP1f and CPRT (5'-TTCGAGCTCTGGAAAAACTGTAGAAACC-3'; the underlined indicates the *Sac*I site) corresponding to nucleotides 6,347 to 6,366. PCR conditions were 94°C for 5 min, then 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR product was first checked by loading for 1% Tris-borate-EDTA agarose gel and was then cloned into pGEMT-easy vector and sequenced. For Northern blot analysis, total RNA was denatured by glyoxalation before the separation; a ³²P-labeled BaMV-specific probe corresponding to the CP region and 3' UTR was used to measure genomic and subgenomic plus-strand RNA (Lin et al. 1996).

Virion purification and CP isolation.

Virions were purified as described previously (Lin and Chen 1991; Lin et al. 1992) from infected *N. benthamiana* plants. CP was further isolated from purified virion by the phenol method as described (Peck et al. 2001).

Protein analysis.

For N-terminal protein sequencing, purified BaMV virions derived from infected leaves of *C. quinoa* or *N. benthamiana* were denatured by heating to 100°C in a water bath and were separated on 12% SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.). Membranes were stained with Ponceau S staining solution for 1 to 3 min, and bands corresponding to the monomer or dimer of BaMV CP or truncated CP were excised and extensively washed with sterile Milli-Q water. Membrane pieces were sequenced by use of an Applied Biosystems Procise-cLC Protein Sequencer at the Core Facilities for Proteomics and Structural Biology Research (CFPSBR, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan).

For 1-D SDS-PAGE assay, an equal volume of 2× Laemmli buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 200 mM DTT or β-mercaptoethanol) was added to the purified BaMV virions. The sample was boiled in a 100°C water bath for 5 min, and CP was separated on 12% SDS-PAGE. Total proteins from infected leaves of bamboo (*Bambusa vulgaris* McClure), *C. quinoa*, or *N. benthamiana* were extracted directly in 2× Laemmli buffer. Briefly, about 0.1 g of freshly infected leaves was ground in liquid nitrogen with use of a mortar and pestle. Then, the powders were trans-

ferred into 1.5-ml pre-chilled Eppendorf tubes and 100 µl of 2× Laemmli buffer was added. After a brief vortexing, protein samples were denatured immediately by boiling them in a 100°C water bath for 5 min and were electrophoresed through a Tris-glycine system. The 1-D gel was stained with PageBlue-Protein staining solution (Fermentas, Vilnius, Lithuania). Protein bands of interest were excised from the gel and were digested with trypsin (Promega, Madison, WI, U.S.A.) and analyzed by matrix-assisted laser desorption ionization-time of flight-based mass spectrometry (MALDI-TOF) at the CFPSBR or by LTQ nanoLC-nanoESI-MS/MS at the Proteomics Core Lab (Institute of Plant and Microbial Biology/Agricultural Biotechnology Research Center, Academia Sinica).

For Western blot analysis, proteins were blotted onto PVDF membrane and were probed with a polyclonal antibody specific to the BaMV CP (anti-BaMV CP) (Lin and Chen 1991) at 1:5,000 and then with peroxidase-labeled goat anti-rabbit antibody (Sigma, St. Louis) at 1:10,000. Signals were visualized using the Enhanced Chemiluminescence Plus kit (Millipore).

For the 2-D electrophoresis assay, dried CP powder, isolated from freshly purified virions by the phenol method as described by Peck and associates (2001), was dissolved directly in rehydration buffer containing 8 M urea, 2% CHAPS, 40 mM DTT, 0.5% 3-10NL strip buffer, and 0.002% (wt/vol) bromophenol blue and was incubated at room temperature for 1 h. Protein concentration was determined by use of the Bio-Rad R_c D_c protein assay kit (BioRad, Hercules, CA, U.S.A.). About 50 µg of CP in 350 µl of rehydration buffer was loaded for rehydration on the 18-cm, nonlinear, pH 3 to 10, immobilized pH gradient strip of the IPGphor unit (GE Healthcare, Piscataway, NJ, U.S.A.). The conditions of isofocusing and 2-D gel electrophoresis are available upon request. In brief, the rapid ramp condition for isofocusing was 100 Kv per h. The strips were equilibrated in a solution of 6 M urea, 50 mM Tris-HCl (pH 8.8), 2% SDS, 30% glycerol, 1% (wt/vol) DTT, and 0.002% (wt/vol) bromophenol blue for 15 min and were then placed into the same solution, except that DTT was replaced with 2.5% (wt/vol) iodoacetamide for another 15 min. Next, the strips were placed onto 12.5% SDS-polyacrylamide gel sealed by a 1% low-melting-point (LMP) agarose solution containing 1% LMP agarose, 25 mM Tris base, 192 mM glycine, 0.1% SDS, and 0.002% (wt/vol) bromophenol blue. Electrophoresis was subsequently performed. The 2-D gels were stained with PageBlue protein-staining solution. Protein spots of interest were excised from the gel and were digested with trypsin (Promega) and analyzed by MALDI-TOF or Thermo Finnegan ProteomeX LTQ nanoLC-nano ESI-MS/MS as mentioned above.

For rCP expression and purification, pET21CP was transformed into the BL21 (DE3) strain of *E. coli*. A 5-ml overnight bacterial culture was inoculated in 500 ml of Luria Broth and was grown at 37°C. With an optical density at 600 nm of about 0.5 to 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1.0 mM, and the cells were grown for a further 16 h at 25°C. Bacteria were collected by centrifugation at 4,000 × g for 20 min and were frozen at -70°C for further assays or were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail) with 5 ml of lysis buffer per gram of pellet. The cells were then disrupted with sonication on ice and underwent centrifugation at 12,000 rpm for 30 min at 4°C. The supernatants were saved for further protein purification by loading onto a Macro-Prep DEAE Cartridge (BioRad) after passing through a 0.45-µm filter. The fractions containing rCP were pooled and concentrated to 1 ml by Amicon Ultra-10 devices (Millipore) before being loaded onto a Sephacryl S-200 column (HiPrep 16/60, GE Healthcare)

for further purification. Proteins were eluted by buffer S (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 5 mM DTT), and the fractions of rCP were pooled and stored at -70°C in buffer S containing 50% glycerol.

Electron microscopy or SSEM.

For negative staining, crude sap from fresh leaves of *C. quinoa* at 8 dpi or systemic leaves of *N. benthamiana* at 20 dpi were collected and dropped on glow-discharged carbon-coated copper grids. Grids were washed twice with Milli-Q water, were stained with 2% uranyl acetate, and were then air-dried and examined on transmission electron microscopy (Philips CM 100 Bio) under 80 KV. For SSEM, similar procedures were adopted, except that the copper grids were precoated with rabbit anti-BaMV virion serum at 1:100 before the crude sap was dropped on the grids.

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LITERATURE CITED

- Abouhaidar, M. G., and Lai, R. 1989. Nucleotide sequence of the 3'-terminal region of *Clover yellow mosaic virus* RNA. *J. Gen. Virol.* 70:1871-1875.
- Banerjee, N., Wang, J. Y., and Zaitlin, M. 1995. A single nucleotide change in the coat protein gene of *Tobacco mosaic virus* is involved in the induction of severe chlorosis. *Virology* 207:234-239.
- Bendahmane, A., Köhn, B. A., Dedi, C., Baulcombe, D. C. 1995. The coat protein of potato virus X is a strain-specific elicitor of Rx1-mediated virus resistance in potato. *Plant J.* 8:933-41.
- Bol, J. F. 2005. Replication of alfalfa- and ilarviruses: Role of the coat protein. *Annu. Rev. Phytopathol.* 43:39-62.
- Chapman, S., Hills, G., Watts, J., and Baulcombe, D. C. 1992. Mutational analysis of the coat protein gene of *Potato virus X*: Effects on virion morphology and viral pathogenicity. *Virology* 191:223-230.
- Choi, J., and Loesch-Fries, L. S. 1999. Effect of C-terminal mutations of *Alfalfa mosaic virus* coat protein on dimer formation and assembly in vitro. *Virology* 260 (1):182-189.
- Culver, J. N., and Dawson, W. O. 1989. Point mutations in the coat protein gene of the *Tobacco mosaic virus* induce hypersensitivity in *Nicotiana glauca*. *Mol. Plant Microbe Interact.* 2:209-213.
- de Assis Filho, F. M., Paguio, O. R., Sherwood, J. L., and Deom, C. M. 2002. Symptom induction by *Cowpea chlorotic mottle virus* on *Vigna unguiculata* is determined by amino acid residue 151 in the coat protein. *J. Gen. Virol.* 83(Pt 4):879-883.
- Endo, S. 1962. Studies on protease produced by thermophilic bacteria. *J. Ferment. Technol.* 40:346-353.
- Gilardi, P., García-Luque, I., and Serra, M. T. 2004. The coat protein of tobamovirus acts as elicitor of both L2 and L4 gene-mediated resistance in *Capsicum*. *J. Gen. Virol.* 85:2077-2085.
- Goulden, M. G., and Baulcombe, D. C. 1993. Functionally homologous host components recognize *Potato Virus X* in *Gomphrena globosa* and potato. *Plant Cell* 5:921-930.
- Huang, Y. L., Hsu, Y. H., Han, Y. T., Meng, M. 2005. mRNA guanylation catalyzed by the S-adenosylmethionine-dependent guanylyltransferase of *Bamboo mosaic virus*. *J. Biol. Chem.* 280:13153-13162.
- Keil, B. 1992. Page 335 in: *Specificity of Proteolysis*. Springer-Verlag Berlin-Heidelberg-New York.
- Knorr, D. A., and Dawson, W. O. 1988. A point mutation in the *Tobacco mosaic virus* capsid protein gene induces hypersensitivity in *Nicotiana glauca*. *Proc. Natl. Acad. Sci. U.S.A.* 85:170-174.
- Kozak, M. 1986. Bifunctional RNAs in eukaryotes. *Cell* 87:481-483.
- Kozak, M. 2002. Pushing the limits of the scanning mechanism for initiation of translation. *Gene* 299:1-34.
- Koenig, R., and Torrance, L. 1986. Antigenic analysis of *Potato virus X* by means of monoclonal antibodies. *J. Gen. Virol.* 67:2145-2151.
- Koenig, R., Stegemann, M. E., Francksen, H., and Paul, H. L. 1970. Protein subunits in *Potato virus X* group. Determination of the molecular weights by polyacrylamide electrophoresis. *Biochem. Biophys. Acta* 207:184-189.

- Koenig, R., Tremaine, J. H., and Shepard, J. F. 1978. In situ degradation of the protein chain of *Potato virus X* at the N- and C-termini. *J. Gen. Virol.* 38:329-337.
- Krol, M. A., Olson, N. H., Tate, J., Johnson, J. E., Baker, T. S., and Ahlquist, P. 1999. RNA-controlled polymorphism in the in vivo assembly of 180-subunit and 120-subunit virions from a single capsid protein. *Proc. Natl. Acad. Sci. U.S.A.* 96(24):13650-13655.
- Li, Y. I., Chen, Y. J., Hsu, Y. H., and Meng, M. 2001a. Characterization of the AdoMet-dependent guanylyltransferase activity that is associated with the N terminus of *Bamboo mosaic virus* replicase. *J. Virol.* 75:782-788.
- Li, Y. I., Shih, T. W., Hsu, Y. H., Han, Y. T., Huang, Y. L., and Meng, M. 2001b. The helicase-like domain of plant potexvirus replicase participates in formation of RNA 5' cap structure by exhibiting RNA 5'-triphosphatase activity. *J. Virol.* 75:12114-12120.
- Lico, C., Capuano, F., Renzone, G., Donini, M., Marusic, C., Scaloni, A., Benvenuto, E., and Baschieri, S. 2006. Peptide display on *Potato virus X*: Molecular features of the coat protein-fused peptide affecting cell-to-cell and phloem movement of chimeric virus particles. *J. Gen. Virol.* 87(Pt 10):3103-3112.
- Lin, M. K., Chang, B. Y., Liao, J. T., Lin, N. S., and Hsu, Y. H. 2004. Arg-16 and Arg-21 in the N-terminal region of the triple-gene-block protein 1 of *Bamboo mosaic virus* are essential for virus movement. *J. Gen. Virol.* 85:251-259.
- Lin, N. S., and Chen, C. C. 1991. Association of *Bamboo mosaic virus* (BaMV) and BaMV-specific electron-dense crystalline bodies with chloroplasts. *Phytopathology* 81:1551-1555.
- Lin, N. S., and Hsu, Y. H. 1994. A satellite RNA associated with *Bamboo mosaic potexvirus*. *Virology* 202:707-714.
- Lin, N. S., Huang, T. Z., and Hsu, Y. H. 1992. Infection of barely protoplasts with *bamboo mosaic virus* RNA. *Bot. Bull. Acad. Sin.* 33:271-275.
- Lin, N. S., Lin, B. Y., Lo, N. W., Hu, C. C., Chow, T. Y., and Hsu, Y. H. 1994. Nucleotide sequence of the genomic RNA of *Bamboo mosaic potexvirus*. *J. Gen. Virol.* 75:2513-2518.
- Lin, N. S., Lee, Y. S., Lin, B. Y., Lee, C. W., and Hsu, Y. H. 1996. The open reading frame of *Bamboo mosaic potexvirus* satellite RNA is not essential for its replication and can be replaced with a bacterial gene. *Proc. Natl. Acad. Sci. U.S.A.* 93:3138-31342.
- Neelman, L., Van Der Kuyl, A. C., and Bol, J. F. 1991. Role of *Alfalfa mosaic virus* coat protein gene in symptom formation. *Virology* 181:687-693.
- Ozeki, J., Hashimoto, M., Komatsu, K., Maejima, K., Himeno, M., Senshu, H., Kawanishi, T., Kagiwada, S., Yamaji, Y., and Namba, S. 2009. The N-terminal region of the *Plantago asiatica mosaic virus* coat protein is required for cell-to-cell movement but is dispensable for virion assembly. *Mol. Plant Microbe Interact.* 22:677-685.
- Peck, S. C., Nühse, T. S., Hess, D., Iglesias, A., Meins, F., and Boller, T. 2001. Directed proteomics identifies a plant-specific protein rapidly phosphorylated in response to bacterial and fungal elicitors. *Plant Cell* 13:1467-1475.
- Revers, F., Gall, O. L., Candresse, T., and Maule, A. J. 1999. New advances in understanding the molecular biology of plant/potyvirus interactions. *Mol. Plant Microbe Interact.* 12:367-376.
- Sambrook, J., Fritsch, F., and Maniatis, T. 2000. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, U.S.A.
- Shintaku, M. H., and Palukaitis, P. 1992. A single amino acid substitution in the coat protein of *Cucumber mosaic virus* induces chlorosis in tobacco. *Plant Cell* 4:751-757.
- Tenllado, F., and Bol, J. F. 2000. Genetic dissection of the multiple functions of alfalfa mosaic virus coat protein in viral RNA replication, encapsidation, and movement. *Virology* 268(1):29-40.
- Tremaine, J. H., and Agrawal, H. O. 1972. Limited proteolysis of *Potato virus X* by trypsin and plant proteases. *Virology* 49:735-744.
- Verchot-Lubicz, J., Ye, C.-M., and Bamunusinghe, D. 2007. Molecular biology of potexviruses: Recent advances. *J. Gen. Virol.* 88:1643-1655.
- Yang, C. C., Liu, J. S., Lin, C. P., and Lin, N. S. 1997. Nucleotide sequence and phylogenetic analysis of *Bamboo mosaic potexvirus* isolate from common bamboo (*Bambusa vulgaris* McClure). *Bot. Bull. Acad. Sin.* 38:77-84.
- Yang, C. D., Liao, J. T., Lai, C. Y., Jong, M. H., Liang, C. M., Lin, Y. L., Lin, N. S., Hsu, Y. H., and Liang, S. M. 2007. Induction of protective immunity in swine by recombinant *Bamboo mosaic virus* expressing foot-and-mouth disease virus epitopes. *BMC Biotechnol.* 7:62.
- Zhang, H., Todderud, E., and Stubbs, G. 1993. Crystallization and preliminary X-ray analysis of *Papaya mosaic virus* coat protein. *J. Mol. Biol.* 234(3):885-887.

AUTHOR-RECOMMENDED INTERNET RESOURCES

ExPaSy Proteomics server's PeptideCutter website:
expasy.org/tools/peptidecutter/peptidecutter_enzymes.html
 MEGA 4 software (<http://www.megasoftware.net/>)
 MyHits Motif scan website: myhits.isb-sib.ch/cgi-bin/motif_scan
 Pôle BioInformatique Lyonnaise network program sequence analysis database: npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html