Functional identification of two minor capsid proteins from Chinese wheat mosaic virus using its infectious full-length cDNA clones

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Full-length cDNA clones of Chinese wheat mosaic virus (CWMV) RNA1 and RNA2 were produced from single reverse transcription PCR reactions and transcripts were shown to be infectious in both wheat and Nicotiana benthamiana. An efficient and reliable agro-infiltration method was then developed for reverse genetic assays in N. benthamiana. Inoculation of infectious cDNA clones resulted in obvious chlorotic symptoms, and CWMV viral genomic RNAs, capsid protein (CP)-related proteins, and typical rod-shaped particles were detectable on the inoculated and upper leaves, similar to those of WT virus. The optimal temperature for virus multiplication was 12 °C, but the optimum for systematic infection in plants was 17 °C. Mutant clones that abolished the N- or C-terminal extensions of the major CP did not inhibit systemic infection or the formation of rod-shaped particles but sometimes modified the symptoms in inoculated plants. These results suggest that the two minor CP-related proteins of CWMV are dispensable for viral infection, replication, systemic movement and virion assembly in plants.

INTRODUCTION

Soil-borne viruses of cereals occur worldwide and are difficult to control (Adams, 1991; Campbell, 1996; Kühne, 2009). At the end of the last century, a soil-borne virus causing wheat mosaic disease in China was characterized and found to be a new species (Ye et al., 1999; Diao et al., 1999). Chinese wheat mosaic virus (CWMV) is now classified in the genus Furovirus, family Virgaviridae, along with Soil-borne wheat mosaic virus (SBWMV), Soil-borne cereal mosaic virus (SBCMV), Japanese soil-borne wheat mosaic virus, Oat golden stripe virus and Sorghum chlorotic spot virus (Adams et al., 2009). All these furoviruses naturally infect cereal plants and are transmitted by an obligate root-infecting fungus-like organism, Polymyxa graminis (order Plasmodiophorales). The viruses retain their infectivity for many years in the resting spores that remain in the soil, which cannot be chemically controlled (Adams et al., 1991, 2009; Diao et al., 1999; Shirako et al., 2000). Whereas SBWMV mainly occurs in North America and SBCMV in Europe, CWMV predominates in Asia. CWMV-infected wheat plants typically have light chlorotic streaking on the young leaves and bright yellow chlorotic streaking or even purple chlorotic stripes on old leaves (Chen, 1993; Ye et al., 1999). Severely infected plants become stunted, wilt and later die. This commonly leads to grain yield losses of 10–30% and, in severe cases, of up to 70% (Chen, 1993).

CWMV has rigid rod-shaped particles with predominant lengths of 140–160 and 260–300 nm and contains a bipartite single-strand positive RNA genome (Diao et al., 1999; Ye et al., 1999; Yang et al., 2001). RNA1 (7147 nt) encodes three proteins required for viral replication and movement, and RNA2 (3564 nt) is predicted to encode four proteins: the major capsid protein (CP, 19 kDa), two minor CP-related proteins (N-CP, 23 kDa; CP-RT, 84 kDa) produced by translation initiation at a non-canonical CUG start codon or occasional read-through of the UGA termination codon, respectively, and a cysteine-rich RNA-silencing suppressor (CRP, 19 kDa) (Diao et al., 1999; Yang et al., 2001; Andika et al., 2013a; Sun et al., 2013a, b). The development of infectious cDNA clones has revolutionized our understanding of many viruses. The lack of such clones for CWMV has severely hampered investigations into the functional roles of the virus-encoded proteins in infection, replication and pathogenicity. However, after a number of unsuccessful attempts by us and other colleagues, we have now produced the first infectious full-length cDNA clones.
of CWMV and conducted a reverse genetics assay to investigate the roles of the major and minor CPs in viral infection and replication in plants.

**RESULTS**

**Infectivity assay of in vitro transcripts from the recombinant plasmids of RNA1 and RNA2**

To analyse the infectivity of the full-length cDNA clones, a mixture (1 : 1) of *in vitro* transcripts from linearized plasmids pCB-T7-R1 and pCB-T7-R2 was mechanically inoculated into the young leaves of wheat cultivar Yannong 22. The method used ensured that these transcripts had no additional non-viral nucleotides. After 6 weeks, the upper leaves developed typical mosaic symptoms, similar to those formed by the WT virus, but there were no symptoms on the mock-inoculated leaves (Fig. 1a). In Western blots using an antibody against purified CWMV particles, three proteins were identified in the upper leaves of plants inoculated with the transcripts or WT virus. These correspond to the major CP (19 kDa) and the two minor CPs (N-CP, 24 kDa; CP-RT, 84 kDa). No signals were detectable from the mock-inoculated leaves (Fig. 1b). Typical rod-shaped particles were observed by electron microscopy in the extracts of upper leaves from plants inoculated with *in vitro* transcripts (Fig. 1c). By Northern blotting, RNA1 and RNA2 were also detected specifically from the upper leaves of plants inoculated with *in vitro* transcripts (Fig. 1d). These results show that the *in vitro* transcripts from pCB-T7-R1 and pCB-T7-R2 are infectious and cause symptoms and the formation of viral particles, similar to the behaviour of WT virus.

**Agro-infiltration assays of the recombinant binary plasmids harbouring the full-length cDNA clones of CWMV genomic RNAs**

Agro-infiltration is a simple, efficient and reliable method for investigations of infectious viral cDNA clones. We therefore produced the recombinant plasmids pCB-35S-R1 and pCB301-35S-R2 in which RNA1 and RNA2 from the binary constructs were under the control of the 35S promoter from cauliflower mosaic virus (CaMV). The constructs were individually transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and used to infiltrate leaves of *Nicotiana benthamiana*, which is an excellent model for plant–pathogen interactions, an excellent target plant for agro-infiltration (Goodin et al., 2008) and an experimental host for CWMV (Andika et al., 2013b). As soon as 14 days after infiltrating a mixture (1 : 1) of *Agrobacterium* transformed with pCB-35S-R1 and pCB35S-R2 into leaves of *N. benthamiana*, the upper leaves of inoculated plants developed numerous chlorotic local lesions (Fig. 2a–c), but infiltration of leaves with pCB-35S-R1 and pCB301 vector or pCB301-35S-R2 and pCB301 did not result in symptoms (data not shown). In Western blot assays of extracts from leaves with chlorotic local lesions using an antibody against purified CWMV particles, three bands migrating at the same rate as those from CWMV...
infected wheat plant were detected (Fig. 2d). Typical rod-shaped particles were also observed by electron microscopy in tissue extracts (Fig. 2e). Northern blot analysis confirmed that RNA1 and RNA2 of CWMV could be detected in the upper leaves and that their migration patterns were similar to those from wheat infected by the WT virus (Fig. 2f). The results show that agro-infiltration with recombinant binary plasmid was an efficient method to deliver infectious cDNA clones and that the behaviour of in vitro transcripts and binary constructs were similar to those of the WT virus in N. benthamiana or wheat plants. Agro-inoculation of wheat was attempted using A. tumefaciens strains GV3101 and AH105 but no systemic infections were detected 4 weeks post-inoculation.

Temperature sensitivity of the infectious full-length cDNA clones

Previous studies have shown that CWMV infects wheat and N. benthamiana at 16°C but not at 24°C (Zhang & Chen, 2005; Andika et al., 2013b). Systemic infection with SBWMV also required temperatures below 20°C (Ohsato et al., 2003), reflecting the adaption of these viruses to plants that often overwinter in cool conditions. To analyse the temperature sensitivity of the infectious full-length cDNA clones, the constructs were agro-infiltrated onto N. benthamiana plants grown at a range of temperatures (12, 15, 17, 20 and 25°C). Two weeks after infiltration, the inoculated and upper leaves were tested by Northern blot assay. Interestingly, in the inoculated leaves the accumulation of viral RNAs was greatest at 12°C, less at 15°C and much less at 25°C (Fig. 3). However, in the upper leaves, the accumulation of viral RNAs was greatest at 17°C and less at 15°C, and there were almost no signs of viral RNA accumulation at lower (12°C) or higher (20 and 25°C) temperatures. Results using the WT virus were similar to those with the infectious clones (data not shown). Taken together, these

Fig. 2. Infiltration assay using a mixture of the recombinant binary constructs pCB-35S-R1 and pCB-35S-R2. (a) Mild green mosaic lesions formed on N. benthamiana leaves 4 weeks after inoculation. From left to right: mock-inoculated N. benthamiana plant, plant inoculated with WT virus, and plant agro-infiltrated with the plasmid mixture, respectively. (b) Enlargement of the boxed part of (a). (c) Enlargement of the boxed part of (b). (d) Western blot assay for CWMV CP in the leaf homogenates. Lane 1, wheat leaves inoculated with WT virus; lane 2, mock-inoculated wheat leaves; lane 3, wheat leaves inoculated with in vitro transcripts of the infectious clones; lane 4, N. benthamiana leaves agro-infiltrated with the plasmid mixture; lane 5, mock-inoculated leaves of N. benthamiana. (e) Electron micrograph of CWMV particles from inoculated plants, negatively stained in 2% phosphotungstate, pH 7.5. Bar, 50 nm, (f) Northern blot analysis. Lane 1, wheat leaves inoculated with WT virus; lane 2, mock-inoculated wheat leaves; lane 3, mock-inoculated leaves of N. benthamiana; lane 4, wheat leaves inoculated with in vitro transcripts of the infectious clones; lane 5, N. benthamiana leaves agro-infiltrated with the plasmid mixture.

Fig. 3. Northern blot assays showing the accumulation of viral RNAs from the infectious cDNA clones in inoculated and upper leaves of N. benthamiana grown at different temperatures, 2 weeks after inoculation. The probes were oligonucleotides complementary to the 3’ terminus of the CWMV RNAs.
results indicated that CWMV replicates best at low temperatures but that the optimal temperature for systemic infection was 17°C.

**Biological activity of the constructs harbouring a series of RNA2 mutants**

Viruses use a range of expression mechanisms to generate a variety of proteins from a compact genome. In addition to subgenomic RNAs (sgRNAs) and 3′-tRNA-like structures, furoviruses also use alternative translational initiation and read-through of the terminal stop codon. To examine the function of the N-CP (24 kDa) and CP-RT (84 kDa) proteins in virus infectivity, five constructs (CWMV RNA2 M1–M5) (Fig. 4a) were produced from plasmid pCB-35S-R2 by PCR-based site-directed mutagenesis. These mutants (CWMV RNA2 M1–M5) were separately combined with the binary construct pCB-35S-R1 and inoculated onto *N. benthamiana* leaves by agro-infiltration. As expected, in plants infiltrated by a mixture of pCB-35S-R1 and pCB-35S-R2, the major CP (19 kDa) and two minor CP-related proteins (N-CP, 24 kDa; CP-RT, 84 kDa) were detectable by Western blot analysis (Fig. 4b); the major CP (19 kDa) and CP-RT (84 kDa) but not N-CP (24 kDa) were detected in plants infiltrated by a mixture of pCB-35S-R1 and CWMV M1 (Fig. 4b); CP-RT (84 kDa) could not be detected in samples infiltrated by mixtures of pCB-35S-R1 and CWMV M2 or M4 (Fig. 4b); and only the major CP (19 kDa) was detected in samples infiltrated by mixtures of pCB-35S-R1 and CWMV M3 or M5 (Fig. 4b). No distinct bands were detectable in the homogenates of mock-inoculated *N. benthamiana* leaves (Fig. 4b). These results indicated that all these mutants of RNA2 remained infectious despite lacking the ability to translate one or both minor CP-related proteins. *N. benthamiana* plants inoculated with a mixture of pCB-35S-R1 and M1 or M2 developed only mild green mosaic symptoms in their upper leaves, while the mixtures containing M3, M4 or M5 gave rather severe yellow mosaic 14 days after infiltration (Fig. 4c, d). In electron microscopy, rod-shaped particles were observed in extracts from the upper leaves of *N. benthamiana* inoculated with mixtures of pCB-35S-R1 and each of the five mutants (Fig. 4e). The morphology of viral particles was indistinguishable among the mutants and the WT virus, indicating that these mutations did not affect viral assembly and suggesting that the two minor CP-related proteins might not be involved in the formation of viral particles.

Transcripts of these mutant constructs were also infectious to wheat and caused mild mosaic symptoms on leaves (Fig. 5a). Both viral RNAs and particles were detectable by reverse transcription PCR (RT-PCR) (Fig. 5b) and electron microscopy in the upper leaves of inoculated plants 4–6 weeks post-inoculation (Fig. 5c). Symptoms appeared to form more slowly on wheat than on *N. benthamiana* and also more slowly than reported for SBWMV in wheat.

**DISCUSSION**

CWMV was identified as a novel member of the genus *Furovirus* 15 years ago (Ye et al., 1999; Diao et al., 1999; Yang et al., 2001). Sequence analysis showed that CWMV and SBWMV (the type member of the genus) only shared about 63 (RNA2) or 75% (RNA1) nucleotide identity and from 62 (CRP) to 84% (replicase) amino acid identity. The genetic diversity suggested that the two furoviruses might have different molecular properties. Infectious cDNA clones of SBWMV have been successfully constructed and initiated the reverse genetics of furoviruses (Yamamiya et al., 2000), but developing infectious cDNA clones of CWMV has proved to be a challenging task. Several full-length clones of different CWMV isolates were constructed from cDNA libraries using a multiple digestion–ligation cloning strategy, but none of them was infectious, for unknown reasons (Diao A-P, Zheng S-L, Chen J-P & Zhang H-M, unpublished). In this study, the full-length cDNA clones were constructed by a single-step PCR and confirmed to be highly infectious by mechanical inoculation of the *in vitro* transcripts or by agro-infiltration of binary plasmids under the control of CaMV 35S promoters. The clones infected not only wheat but also *N. benthamiana*, an excellent experimental model dicot plant, suggesting that they could be used to develop a universal artificial vector of expression or virus-induced gene silencing (VIGS) from monocot to dicot plants. The deletion of a large fragment of RNA2 (nt 1098–2540) did not appear to affect the infectivity of the cDNA clones, which suggested that the constructs could allow the insertion of more than 1.5 kb fragments for expression or VIGS. The agro-infiltration system will also provide a quick and easy method for developing the reverse genetics of CWMV and for identifying virus resistance in different host plants.

Non-AUG translation initiation has been reported for both viral and cellular gene expression and its efficiency depends on the context, just as for the AUG codon (Kozak, 1989; Joshi et al., 1997). In plants, CUG was the next most efficient initiator after AUG (Gordon et al., 1992), and it is used by many viruses for regulation of viral gene diversity and expression (Shirako et al., 1998; Zhou et al., 2006; Firth et al., 2009; Sun et al., 2013b). In CWMV, use of an upstream in-frame CUG codon produces an extended form of the CP (N-CP), but its expression appears to be much less than that of CP (Figs 1b and 2d) in both wheat and *N. benthamiana*, indicating a lower efficiency in CUG translation initiation despite a fairly good Kozak context (Kozak et al., 1989; Sun et al., 2013b). Both mutants lacking the expression of N-CP (CWMV M1 and M3) were able to replicate and systemically infect plants, similar to those of SBWMV (Yamamiya et al., 2000). However, the single mutation of the non-canonical start codon (CWMV M1) appeared to slow symptom development (Fig. 4c, d), as also reported for SBWMV and *Hibiscus* chlorotic ringspot virus (genus *Carmovirus*) (Yamamiya et al., 2000; Zhou et al., 2006), suggesting that the translational control of viral N-terminally extended proteins may reflect a general
Fig. 4. (a) Schematic diagram of the five mutant constructs from binary plasmid pCB-35S-R2. CWMV (WT), pCB-35S-R2; CWMV M1, the CTG initiation codon for N-CP was replaced with CAG; CWMV M2, another TGA termination codon was added in place of the WT Arg codon (CGG) adjacent to the TGA termination codon for CP; CWMV M3, mutations in M1 and M2 were combined; CWMV M4, the WT CGG codon adjacent to the TGA was replaced with TGA termination codon for CP and the region nt 1098–2540 was deleted; CWMV M5, mutations in M3 and M4 were combined. RT, Read-through domain, K, molecular weight of proteins (kDa). (b) Western blot showing detection of major and/or minor CPs expressed from WT and five mutant constructs. (c–e) Symptom expression (c, d) and virus particles (e) of the five CWMV mutants M1–M5. The lower row images (d) are enlargements of the boxed areas in the photos immediately above (c). Bars, 50 nm.
mechanism that regulates symptom expression. It has recently been reported that the N-CP protein can be detected in purified CWMV virions, suggesting that it is incorporated into particles (Sun et al., 2013b). However, the particles of the WT virus were indistinguishable from the mutants lacking the expression of N-CP (Fig. 4b, e), suggesting that the N-CP is not required for the formation of virus particles even if it is incorporated into them.

Viral CP-RT proteins have been studied in a number of rod-shaped viruses transmitted by plasmodiophorids. The CP-RTs of beet necrotic yellow vein virus (BNYVV; genus Benyvirus) and potato mop-top virus (PMTV; genus Pomovirus) are associated with one extremity of the viral particles (Cowan et al., 1997; Haeberle et al., 1994). BNYVV CP-RT is required for virus transmission and is important for efficient virus assembly (virion

Fig. 5. Symptom expression (a) and detection of viral RNAs by RT-PCR (b) and particles by electron microscopy (c) in the upper leaves of wheat plants inoculated with transcripts of the five CWMV mutants M1–M5. Bars, 50 nm.
morphogenesis) and mitochondrial localization (Tamada et al., 1991, 1996; Schmitt et al., 1992; Erhardt et al., 2001; Valentin et al., 2005); PMTV CP-RT is required for symptom production but is dispensable for viral infection and replication (Torrance et al., 2009). Interestingly, the systemic movement of PMTV particles could be mediated by interaction of CP-RT, but not CP, with TGB1 (Torrance et al., 2009). CWMV CP-RT was detected on the surface of virus particles in infected wheat sap and leaf tissue by immuno gold labeling with specific antisera or mAbs against the read-through (RT) domain (Xu et al., 2002), suggesting that CP-RT could be associated with the virus particles. Paradoxically, no signal of CP-RT was found by Western blot analysis of purified CWMV virions (Sun et al., 2013b). In this study, the virus particles of the WT virus and mutants lacking the expression of CP-RT were indistinguishable (Fig. 4b, e), indicating that the CP-RT was dispensable for the formation of viral particles and systemic movement in host plants, as has been reported for SBWMV (Yamamiya et al., 2000; Shirako et al., 2000) and beet soil-borne virus (genus Pomovirus) (Crutzen et al., 2009).

The read-through domain of CP-RT has two highly conserved transmembrane domains, which are thought to be associated with virus transmission by facilitating the movement of virus particles across the plasmodiophorid membrane. Spontaneous mutants in which these domains are deleted can no longer be transmitted by the vector (Adams et al., 2001). Further identifying the biological properties of these CP-RT mutants could provide a deeper insight into the transmission mechanism.

Beside its putative role in transmission, CWMV CP-RT appeared to affect symptom development in host plants. In this study, the mutants M3–M5 (which abolished the translation of the RT domain or deleted its encoding region (Fig. 4b), caused serious symptoms (Fig. 4c, d) similar to those of the spontaneous deletion mutant of SBWMV CP-RT (Chen et al., 1994). However, the M2 mutant (which retained the N-CP but abolished the translation of the RT domain), and the M1 mutant (which only abolished the N-CP; Fig. 4) appeared to slow symptom expression (Fig. 4c, d). These results suggested that there may be some form of direct/indirect interaction or balance between the N-CP and RT-CP proteins that regulates symptom expression.

### METHODS

**Virus isolate and viral RNA extraction.** CWMV-infected wheat plants with typical mosaic symptoms were collected from a disease nursery in Yantai city, Shandong province, PR China. Virions were purified as described previously (Diao et al., 1999) and the genomic RNAs were extracted from them using Trizol reagent (Invitrogen) and stored at −80 °C.

**Construction of recombinant plasmids.** The viral RNAs were used as templates for reverse transcription with Superscript III (Invitrogen) and then amplified by PCR with Phusion High-Fidelity DNA Polymerases (NEB) using primer pair P1F/ P1R or P2F/P2R (Table 1). The resultant 7.2 kb PCR product of full-length CWMV RNA1 was digested with BamHI/SacI and ligated into the BamHI/SacI-digested plasmid pCB301 vector (GenBank accession number JN029690), resulting in pCB-T7-R1. The 3.6 kb PCR product of full-length CWMV RNA2 was digested with HindIII/EcoRI and ligated into the HindIII/EcoRI-digested plasmid pCB301 to obtain pCB-T7-R2. The resultant plasmids pCB-T7-

### Table 1. Primers used for construction of infectious full-length cDNA clones

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Target fragment</th>
</tr>
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<tbody>
<tr>
<td>P1F*</td>
<td>CGGGACCTTTAAATACGACTCTACTATAGTTATTCTTCTCTACGTGGTTAG</td>
<td>CWMV RNA1 fused with T7 promoter at 5′ end</td>
</tr>
<tr>
<td>P1R†</td>
<td>ACGAGCCTCCAGGTTGGGCGGAATAACCTCCG</td>
<td>CWMV RNA2 fused with T7 promoter at 5′ end</td>
</tr>
<tr>
<td>P2F‡</td>
<td>CCGAACGTCTTTAATACGACTCTACTATAGTTATTCTTCTCTACGTGGTTAGTG</td>
<td>CWMV RNA1 fused with T7 promoter at 5′ end</td>
</tr>
<tr>
<td>P2R§</td>
<td>GGAATTCCGCGTTGCGCCGGTTTACCACCCACC</td>
<td>Binary plasmid pCB301</td>
</tr>
<tr>
<td>pCB301F</td>
<td>GGGTGCGGCTAGGCTATCTCG</td>
<td>pCB301</td>
</tr>
<tr>
<td>pCB301R</td>
<td>CCTCCTCCAAATGAAATGAGCTTCC</td>
<td>pCB301</td>
</tr>
<tr>
<td>P3F</td>
<td></td>
<td>TTTCTATTGGACAGAGGTATTTTCTCTCTACGTGGTTCGAC</td>
</tr>
<tr>
<td>P3R*</td>
<td>ATGCCATGCCACCTTGCGCCGGATAACCTCC</td>
<td>CWMV RNA1</td>
</tr>
<tr>
<td>P4F#</td>
<td>TTTCTATTGGACAGAGGTATTTTCTCCTACGTGGTTCGAC</td>
<td>CWMV RNA1</td>
</tr>
<tr>
<td>P4R**</td>
<td>ATGCCATGCCACCTTGCGCCGGTTTACCACCCACC</td>
<td>CWMV RNA1</td>
</tr>
</tbody>
</table>

*BamHI site in bold; T7 promoter underlined.
†SacI site in bold; MluI site underlined; italicized letters, 19 nt complementary to the 3′-terminal region of RNA1.
‡HindIII site in bold; T7 promoter underlined; italicized letters, 5′-terminal 26 nt of RNA2.
§EcoRI site in bold; MluI site underlined; italicized letters, 18 nt complementary to the 3′-terminal region of RNA2.
||Underlined letters, 15 nt of vector pCB301 positions 753–767; italicized letters, 5′-terminal 28 nt of RNA1.
‡Underlined letters, 15 nt complementary to vector pCB301 positions 800–814; italicized letters, 17 nt complementary to the 3′-terminal region of RNA1.
#Underlined letters, 15 nt of vector pCB301 positions 753–767; italicized letters, 5′-terminal 26 nt of RNA2.
**Underlined letters, 15 nt complementary to vector pCB301 positions 800–814; italicized letters, 18 nt complementary to the 3′-terminal region of RNA2.
R1 and pCB-T7-R2 were then transformed into Turbo *Escherichia coli* (NEB). Three clones of each transformation with plasmids pCB-T7-R1 or pCB-T7-R2 were sequenced, confirming that they contained the full-length RNA1 or RNA2 of CWMV.

For agro-infiltration, the plasmids pCB-T7-R1 and pCB-T7-R2 were then modified using the In-Fusion Cloning kit (Clontech) to make constructs under transcriptional control of the 35S promoter according to commercial protocols. In brief, the plasmid pCB301 was used as a template for PCR amplification using primer pair pCB301F/pCB301R to obtain the linearized vector. The pCB301 vector contains, in sequential order, a left border of T-DNA (LB), a double 35S promoter (35S), an HDV cis-cleaving ribozyme sequence (Rz), a NOS terminator (Nos), and a right border of T-DNA (RB). The CWMV cDNA clones were positioned between a truncated CaMV double 35S promoter (2×35S) and a self-cleaving hepatitis deltavirus ribozyme sequence to ensure synthesis of CWMV genomic RNA transcripts with exact 5¢ and 3¢ ends. DNA corresponding to the full-length of CWMV RNA1 or RNA2 (200 ng) was then amplified with primer pairs P3F/P3R or P4F/P4R and digested into the linearized pCB301 vector (50 ng) using In-Fusion HD Enzyme Premix (Clontech). The resultant plasmid pCB-35S-R1 or pCB-35S-R2 was transformed into Turbo *E. coli* (NEB) and three clones of each were verified by sequencing. All the primers are shown in Table 1.

To study the functions of the major and minor CPs, five mutant constructs (M1–M5) (Fig. 4a) were generated from plasmid pCB-35S-R2 using PCR-based site-directed mutagenesis with the primer pairs listed in Table 2. These constructs abolished the translation of the N-terminal extension of CP (N-CP, 24 kDa), the RT domain of the CP (CP-RT, 84 kDa), or both (Fig. 4).

**In vitro transcription.** The recombinant plasmids pCB-T7-R1 and pCB-T7-R2 were linearized with SpeI restriction digestion immediately downstream of the 3¢ terminus of CWMV RNA1 and RNA2 and purified by repeated phenol/chloroform extractions and ethanol precipitation. The linearized plasmids were then used as DNA templates for in vitro transcription of CWMV RNA1 and RNA2, respectively. The T7-capped transcription reactions were performed with the Ambion Message Machine kit (Invitrogen) according to the manufacturer’s protocols. After adding approximately 0.1 µg of linearized plasmid templates, the reaction mixtures were incubated at 37 ºC for 1 h and then 1 µl of reaction products was analysed for the quantity and integrity of the transcripts by electrophoresis in a 1.0 % agarose gel.

**Infectivity assay of in vitro transcripts.** Mixtures of in vitro transcripts from RNA1 and RNA2 were used for infectivity assay as previously described (Yamamiya *et al.*, 2000). In brief, approximately 5 µg of each transcript was suspended in 1 ml 50 mM glycine and 50 mM K2 HPO4 (pH 9.2), and then mechanically inoculated onto leaves of wheat cultivar Yannong 22 (2 weeks old), a susceptible cultivar, and *N. benthamiana* leaves (4 weeks old) using Celite as an abrasive. Inoculated plants were maintained in a growth cabinet at 17 ºC unless stated otherwise.

**Western blot analysis.** About 0.1 g leaf tissue frozen in liquid nitrogen was ground to a fine powder and thawed in plant protein extraction buffer (Sigma) containing Protease Inhibitor Cocktail tablets (1 tablet per 50 ml; Roche). The mixture was centrifuged at 18 000 g for 10 min at 4 ºC. Twenty micrograms of each protein sample was boiled in SDS-PAGE buffer at 100 ºC for 1 h and then centrifuged at 18 000 g for 5 min. The supernatant was re-dissolved in 15 % SDS-PAGE buffer, followed by Western blot analysis using rabbit polyclonal antibody against purified CWMV particles as the primary antibodies (1:5000) and anti-rabbit goat IgG conjugated with alkaline phosphatase as the secondary antibody (1:10 000) following the method described previously (Yang *et al.*, 2014).

**Northern blot assays.** Total RNAs were extracted from leaf tissues using TRIzol reagent (Invitrogen) and treated with DNase (TaKaRa). Their aliquots (3 µg) were separated on a denaturing 2 % formaldehyde agarose gel and transferred to Hybond-N+ membranes (Amersham Biosciences) using 20× SSC (saline-sodium citrate) buffer. The RNAs were cross-linked to membrane matrix by UV for 45 s. Northern blotting for assays of CWMV genomic RNAs was carried out using the DIG High

| Table 2. Primers used for construction of five CWMV mutants M1–M5 |
|-------------------------|---------------------------|---------------|
| **Primers**             | **Sequence** (5¢–3¢)       | **Mutant(s)** |
| 3SSF†                   | CCCAGCTTGGCATGCGTCCAGTC   | M1            |
| P5R§                    | GCTACTGAACTATCCTGGACGCGTAACACCGAGTTACTCC | M1            |
| P6F§                    | GAGTAACTGCTTACGGCTGCCAGATGGTTCAGTGGC   | M1            |
| P6R||                   | GCTGGTGTCCTCCTCTTC         | M1            |
| P7¶                     | GCAGTCTAGAATGGCGAAGAGGAG  | M2 and M3    |
| P7R#                    | CTAATCAACTCGAACCCTTCCAGTTAG | M2 and M3    |
| P8F**                   | GGTTCGAGTTGAGAGAGAAGAGGAG | M2 and M3    |
| P8R††                   | GACGATGACGAACTGAACAGTTAGAAG | M3            |
| P9F‡‡                   | gACTAGTTGCTGGAGGAAAGGTGTTGAGTAG | M4 and M5 |
| P9R$$$                  | GAGATCAGTGCTGGCCTGCTGCTG | M4 and M5    |

*Mutated bases are shown in italic letters and the restriction digestion sites in bold letters.
†3SSF used to amplify the 35S promoter of the pCB301 vector; HindIII site underlined.
‡Positions 184–225 in the minus strand of CWMV RNA2; T to A substitution at position 208 is indicated in italic type.
§Positions 184–225 in the plus strand of CWMV RNA2; A to T substitution at position 208 is indicated in italic type.
||Positions 644–669 in the plus strand of CWMV RNA2.
#Positions 831–854 in the minus strand of CWMV RNA2; the additional stop codon CTA (reverse complement of TAG) at position 854.
**Positions 843–855 in the plus strand of CWMV RNA2; the additional stop codon TAG at position 854.
††Positions 1186–1211 in the minus strand of CWMV RNA2.
‡‡Positions 2541–2563 in the plus strand of CWMV RNA2; SpeI site in bold.
§§Positions 2989–3101 in the plus strand of CWMV RNA2.
Prime DNA Labeling and Detection Starter Kit II (Roche). The DNA oligonucleotides complementary to the 3’ terminus of the CWMV genome were labelled with digoxigenin (DIG) at their 3’ ends using the DIG Oligonucleotide Tailing kit (Roche) and then purified using a G25 Sephadex column (GE). Membranes were pre-hybridized for 2 h and hybridized overnight at 42°C using the DIG Luminescent Detection Starter kit for nucleic acids (Roche). The hybridization signals were visualized by using an Amersham Imager 600 (GE). All these procedures were performed according to the manufacturer’s instructions.

**Electron microscopy.** Wheat leaves showing typical mosaic symptoms and *N. benthamiana* leaves showing chlorotic local lesions were frozen in liquid nitrogen, ground to fine powder and thawed in phosphate buffer. The sap was placed on a grid with a collodion film pretreated with rabbit polyclonal antibody against CWMV virions (diluted 1 : 1000) for 45 min. After washing three times with droplets of water, the grid was treated with phosphotungstic acid (pH 7.5), and examined under an H-7650 transmission electron microscope (Hitachi) at 80 kV accelerating voltage. Photographs were taken with a Gatan 830 CCD camera.

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