The Red clover necrotic mosaic virus capsid protein N-terminal lysine-rich motif is a determinant of symptomatology and virion accumulation

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SUMMARY

The interaction between viral capsid protein (CP) and its cognate viral RNA modulates many steps in the virus infection cycle, such as replication, translation and assembly. The N-terminal 50 amino acids of the Red clover necrotic mosaic virus (RCNMV) CP are rich in basic residues (especially lysine) and are essential for the core functions of the CP, namely RNA binding and virion assembly. To further elucidate additional biological roles for these basic residues, a series of alanine substitution mutations was introduced into infectious clones of RCNMV RNA-1 and assayed for symptomatology, virion formation and systemic infection. Infectivity assays conducted in *Nicotiana benthamiana* revealed that all nine alanine substitution mutants (ASMs) were competent for systemic infection. Two ASMs (K4A and K7A/K8A) induced severe symptoms and delayed the systemic spread of viral genomes when compared with wild-type RCNMV. However, these ASMs were still competent for virion formation. Three other ASMs (K25A, K33A and K38A) displayed milder symptoms and significant reductions in virion accumulation when compared with wild-type RCNMV, but retained the ability to spread systemically. Evidence from these last three ASMs, as well as a CP null mutant, showed that RCNMV is able to move systemically in *N. benthamiana* as a nonvirion form. These observations reaffirm the necessity of the N-terminal lysine-rich residues of the RCNMV CP for efficient virion accumulation. They also reveal additional roles for the CP in the modulation of host symptomatology, independent of its role in virion assembly and the rate of systemic viral movement in *N. benthamiana*.

INTRODUCTION

Plant virus capsid proteins (CPs) are involved in multiple functions in addition to their primary structural role, such as the translation of viral RNA, targeting of the viral genome to its site of replication, cell-to-cell and/or systemic movement, symptomatology/virulence

of the infection, activation of *R* gene-mediated host defences, suppression of RNA silencing and determination of vector specificity for virus transmission (Bol, 2008; Pallas and Garcia, 2011). As a result of this variety of functions, many amino acid residues in the CP probably affect pathogenesis on a particular host, either directly or indirectly. For example, the Turnip crinkle virus (TCV, genus *Carmovirus*) CP is implicated in virus movement and symptom expression (Hacker *et al.*, 1992; Heaton *et al.*, 1991). The N-terminal 25 amino acids of the TCV CP are also involved in the ability to suppress RNA silencing, and have been identified as the elicitor for TIP (TCV-interacting protein) expression which confers the hypersensitive response (HR) in *Arabidopsis thaliana* (Qu *et al.*, 2003; Ren *et al.*, 2000; Thomas *et al.*, 2003; Zhao *et al.*, 2000).

Red clover necrotic mosaic virus (RCNMV), a species in the *Dianthovirus* genus in the *Tombusviridae* family, is a plus sense and single-stranded RNA virus possessing two genomic RNAs: RNA-1 and RNA-2. RNA-1 encodes p27 and p88 (virally encoded components of the replicase complex) and the structural CP (Xiong and Lommel, 1989). RNA-2 encodes for the movement protein (MP) which is required for cell-to-cell movement (Xiong *et al.*, 1993). In addition, RNA-2 contains the transactivator (TA) element within the MP open reading frame (ORF) that interacts with the TA-binding site (TABS) on RNA-1 to initiate subgenomic RNA (sgRNA) synthesis for CP production (Sit *et al.*, 1998). Although RCNMV virions share the same morphology as other species in the *Tombusviridae*, such as Tomato bushy stunt virus (TBSV), TCV and Carnation mottle virus (CarMV) (Musil and Gallo, 1982), the encapsidation mechanism must be different and more complex because of the bipartite nature of the RNA genome. Recent studies on the RCNMV packaging mechanism have revealed that virions are divided into two distinct populations containing either one copy of each genomic RNA or four copies of RNA-2 only (Basnayake *et al.*, 2006). Furthermore, the TA element on RNA-2 has been determined to be the origin of assembly sequence (OAS; Basnayake *et al.*, 2009). For precise assembly, the RCNMV packaging signal must be distinguished from other viral and cellular RNAs by interacting with the cognate RNA-binding region of the CP. We have demonstrated previously that the 50 N-terminal residues of the RCNMV CP are highly basic (eight lysine

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and four arginine residues) and are required for both viral RNA binding and virion formation (S.-H. Park *et al.*, unpublished data). To uncover any other biological roles for this RNA-binding region during viral infection, a series of site-directed mutants were engineered and their effects were tested on the systemic host *Nicotiana benthamiana*. The results obtained suggest that the basic residues within the RCNMV CP RNA-binding motif play an important role in symptom development and virion accumulation.

RESULTS

Symptomatology is altered by mutations to the RCNMV CP N-terminal basic residues

The *in vitro* RNA-binding activity of the RCNMV CP N-terminal 50 residues prompted us to test whether the basic residues within this region affected other processes in addition to encapsidation. Although the initiation of assembly is highly specific, other less specific RNA interactions must occur to package the rest of the genome, such as the dampening of negatively charged phosphate groups on the RNA by electrostatic interaction with positively charged amino acids (S.-H. Park *et al.*, unpublished data). The N-terminal region of the RCNMV CP is rich in basic residues, especially lysine, and we refer to this region as the lysine-rich RNA-binding motif (LRM). Alignment of the RCNMV CP LRM with that of the other two dianthoviruses, *Sweet clover necrotic mosaic virus* (SCNMV) and *Carnation ringspot virus* (CRSV), revealed that most of the basic residues are highly conserved and that the N-terminal amino acid sequence of RCNMV is more similar to that of SCNMV than that of CRSV (Fig. 1A).

Alanine substitution mutations of these basic residues were introduced into the LRM of an infectious full-length RCNMV RNA-1 construct (pRC169; Fig. 1B). *In vitro* transcripts of either wild-type (WT) or CP alanine substitution mutant (ASM) RNA-1 constructs were mechanically co-inoculated with WT RNA-2 transcripts onto the fifth and sixth leaves of *N. benthamiana* plants and observed daily for symptom development. At 7 days post-inoculation (dpi), mosaic symptoms were observed on upper non-inoculated leaves of WT RCNMV transcript-inoculated plants. Although mutants K10A/R12A and R48A displayed no obvious difference in symptomatology when compared with WT (Fig. 2A,E), all other ASMs showed altered symptomatology. The foremost difference was the severe systemic necrosis produced by mutants K4A and K7A/K8A (Fig. 2A,E). Mutant R16A/R18A showed more severe symptoms (severe mosaic and necrosis) than WT RCNMV, but less severe symptoms than mutants K4A and K7A/K8A. The remaining four ASMs (K25A, K33A, K38A and K45A) induced mosaic symptoms which were milder than those of WT RCNMV (Fig. 2A,E).

Passaging experiments were performed by sap inoculation to determine the stability of the altered symptomatology for five ASMs. Inoculation of sap extracts from mutants K4A and K7A/K8A showed dramatically more severe necrotic symptoms (as expected) when compared with the milder mosaic symptoms produced by mutants K25A, K33A and K38A (Fig. 3A,B). The full-length genomic sequences of progeny viruses were determined, and no new alterations or reversions in amino acid sequence were detected (data not shown). These results indicate that CP LRM ASMs are stable and produce significant alterations to host symptomatology.

Fig. 1 The alignment of the coat protein (CP) N-terminal 50 amino acids of three dianthoviruses and schematic diagram of the full-length cDNA clones of *Red clover necrotic mosaic virus* (RCNMV) RNA-1, CP mutants and RNA-2. (A) Alignment analysis of the N-terminal RNA-binding motif of RCNMV CP with the other two dianthoviruses: *Sweet clover necrotic mosaic virus* (SCNMV) and *Carnation ringspot virus* (CRSV). (B) Schematic representation of the RCNMV genome organization and position of alanine substitution mutations within the CP. The -1 frameshift site (-1 FS) responsible for the translation of p88 is shown. The transactivator (TA) on RNA-2 and the TA-binding site (TABS) on RNA-1 are depicted as black boxes. The substituted residues are denoted by arrows with their position numbers. nt, nucleotide. WT, wild-type.

Fig. 2 Effect of basic amino acid substitutions in the coat protein (CP) on symptom development and viral RNA accumulation. (A–D) Results from inoculated leaves at 4 days post-inoculation (dpi). (E–H) Results from upper noninoculated leaves at 7 dpi. (A, E) Symptom development on leaves of *Nicotiana benthamiana*. (B, F) Viral RNA accumulation analysed by Northern blotting. Viral RNA-1 (C, G) and RNA-2 (D, H) accumulation measured by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis. Each value of qRT-PCR is the mean and standard error relative to that of the wild-type (WT) compiled from at least three independent experiments for both RNA-1 and RNA-2 accumulation.

Fig. 3 Passage experiments with sap extracts from wild-type (WT) and five alanine substitution mutant (ASM) transcripts inoculated onto *Nicotiana benthamiana*. Symptom appearance on sap-inoculated plants is shown from the side (A) and above (B).

Inverse correlation between the severity of systemic symptomatology and viral RNA accumulation levels

Northern blot and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses were performed to determine whether the symptoms induced by ASMs correlated

with viral RNA accumulation levels. After the WT RCNMV and nine ASMs had been inoculated onto *N. benthamiana*, total RNAs were extracted from inoculated leaves at 4 dpi and from upper noninoculated leaves (the third leaf from the top) at both 4 and 7 dpi. Northern blot and qRT-PCR results from inoculated leaves indicated that mutants K4A and K7A/K8A were found, in general, to show increased viral RNA accumulation levels (with the exception of RNA-1 for K7A/K8A which showed decreased accumulation), whereas mutants K25A, K33A and K38A displayed significantly reduced levels of both viral genomic RNAs when compared with WT RCNMV (Fig. 2B–D). The observations were reversed in the upper noninoculated leaves, where viral genomic RNAs of mutants K4A and K7A/K8A (which elicited severe necrotic symptoms) accumulated to lower levels, whereas those of mutants K33A and K38A (which induced milder symptoms) accumulated to higher levels, than those of WT RCNMV (Fig. 2F–H). Similar or lower levels of accumulation were detected in *N. benthamiana* tissues inoculated with the other ASMs (Fig. 2A–H and summarized in Table 1). As illustrated in Fig. 4 and Table 2, qRT-PCR results from the upper noninoculated leaves at 4 and 7 dpi showed that the differences in accumulation between K4A, K7A/K8A, K33A and K38A became more pronounced as time elapsed. Unlike mutants K33A and K38A, mutant K25A did not show a dramatic increase in RNA accumulation in the upper noninoculated leaves despite the induction of similar mild symptoms. This suggests that other factors in addition to the RNA accumulation level affect the symptomatology induced by mutant K25A. Overall, the results produced by these ASMs suggest that single or double amino acid conversions within the CP LRM result in dramatic changes in symptomatology, which are correlated inversely with (but may not be attributable to) viral RNA accumulation levels.

Systemic movement does not require virion formation

The results obtained above demonstrate that ASMs can alter the symptomatology and RNA accumulation levels. We next examined whether these observations were the direct result of changes to virion accumulation efficiency caused, perhaps, by disruption of the CP–RNA interaction. Previous work has concluded that virion formation is required for systemic movement in *N. benthamiana* (Vaewhongs and Lommel, 1995). Virions were purified from inoculated and upper noninoculated leaves of *N. benthamiana* at 4 and 7 dpi, respectively. Agarose gel electrophoresis and transmission electron microscopy (TEM) were used to assay for virion formation. Purified virions were electrophoresed in agarose gels under nondenaturing conditions and stained with either ethidium bromide or Coomassie brilliant blue R250 (CBB-R250) (Pappu and Hiruki, 1989). Gel electrophoresis revealed that ASM samples which contained virions migrated similarly to that of WT RCNMV (Fig. 5A–D). However, no virions

Mutant	Symptoms*	Relative quantity†			
		Inoculated		Noninoculated	
		RNA-1	RNA-2	RNA-1	RNA-2
WT	m	1.00 ± 0.14	1.00 ± 0.17	1.00 ± 0.14	1.00 ± 0.23
K4A	sm, sn	1.78 ± 0.34	2.37 ± 0.55	0.30 ± 0.34	0.45 ± 0.04
K7A/K8A	sm, sn	0.64 ± 0.15	2.98 ± 0.65	0.11 ± 0.15	0.44 ± 0.08
K10A/R12A	m, sm	1.16 ± 0.22	0.74 ± 0.15	0.32 ± 0.13	0.52 ± 0.18
R16A/R18A	sm, sn	1.07 ± 0.29	1.55 ± 0.21	1.06 ± 0.10	0.35 ± 0.10
K25A	mm	0.11 ± 0.03	0.11 ± 0.04	0.98 ± 0.15	0.96 ± 0.18
K33A	mm	0.42 ± 0.13	0.27 ± 0.05	4.58 ± 0.85	4.08 ± 0.26
K38A	mm	0.19 ± 0.08	0.13 ± 0.02	5.33 ± 1.56	5.28 ± 1.71
K45A	mm	0.65 ± 0.13	0.67 ± 0.15	0.34 ± 0.12	0.71 ± 0.27
R48A	m	1.29 ± 0.38	1.12 ± 0.37	0.25 ± 0.12	0.48 ± 0.27

*Symptom development was observed at 14 days post-inoculation (dpi). m, mosaic; mm, mild mosaic; sm, severe mosaic; sn, systemic necrosis.

†RNA relative quantity from inoculated (4 dpi) and noninoculated (7 dpi) leaves was estimated by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR).

Table 1 Summary of *Red clover necrotic mosaic virus* (RCNMV) coat protein (CP) alanine substitution mutants (ASMs) in Fig. 2.

Fig. 4 Analysis of viral genomic RNA accumulation by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) in upper noninoculated leaves at 4 days post-inoculation (dpi) (A) and 7 dpi (B). qRT-PCR was performed as described in Fig. 2.

Table 2 Summary of *Red clover necrotic mosaic virus* (RCNMV) coat protein (CP) alanine substitution mutants (ASMs) in Fig. 4.

Mutants	Relative quantity			
	4 dpi		7 dpi	
	RNA-1	RNA-2	RNA-1	RNA-2
WT	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.20	1.00 ± 0.21
K4A	0.66 ± 0.04	0.96 ± 0.04	0.43 ± 0.05	0.64 ± 0.06
K7A/K8A	0.47 ± 0.03	1.21 ± 0.08	0.54 ± 0.14	0.84 ± 0.14
K25A	0.53 ± 0.09	1.21 ± 0.15	1.60 ± 0.27	1.98 ± 0.23
K33A	1.09 ± 0.07	1.53 ± 0.06	7.21 ± 1.56	6.80 ± 1.31
K38A	1.18 ± 0.22	1.69 ± 0.16	6.99 ± 1.74	7.83 ± 2.16

RNA relative quantity from noninoculated leaves [4 and 7 days post-inoculation (dpi)] was estimated by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR).

were observed in preparations from mutants K25A, K33A and K38A (Fig. 5A–D). Western blot analysis with virion preparations revealed that these three ASM samples showed detectable levels of CP only when substantially greater amounts of virions were loaded in comparison with WT RCNMV or mutants K4A and K7A/K8A (Fig. 5E,F). Next, Western blot analysis with total protein extracts was performed to examine the effect of mutations on CP accumulation and stability. The stability and accumulation of the mutated CP were not affected by mutations when compared with that of WT in inoculated and noninoculated leaves (Fig. 5G,H). To corroborate these findings, TEM analysis revealed that mutants K4A, K7A/K8A, K10A/R12A, R16A/R18A, K45A and R48A were morphologically indistinguishable from WT virions, but virions were not detected from preparations of mutants K25A, K33A and K38A (Fig. 6A). Viral RNAs were extracted from all virion preparations and subjected to Northern blot hybridization analysis with probes corresponding to the 3' region of both gRNAs. Virion RNAs from mutants K25A, K33A and K38A were significantly reduced in both inoculated and upper noninoculated leaves (Fig. 6B,C). Taken together, these results demonstrate that mutations at positions 25, 33 and 38 drastically reduce virion formation and symptomatology, whilst still allowing robust systemic movement.

To definitively confirm that RCNMV can move systemically without virion formation, *in vitro* transcripts of the CP-null mutant (possessing an ATGTCT to ATCGAT mutation at the CP initiation codon that prevents CP expression) were co-inoculated with WT RNA-2 transcripts onto *N. benthamiana*. The CP-null mutant displayed very mild symptoms when compared with the WT virus (Fig. S1A, see Supporting Information), but moved systemically and accumulated viral RNAs in the upper noninoculated leaves, albeit at reduced levels when compared with the WT virus (Fig. S1B). This result indicates that, consistent with the results obtained with mutants K25A, K33A and K38A, systemic movement of RCNMV can occur in the absence of virion formation.

DISCUSSION

We have demonstrated here that basic residues in the RCNMV CP LRM are symptomatology and assembly determinants. The LRM consists of 50 residues (including eight lysines and four arginines), none of which are acidic and are highly conserved among the dianthoviruses (Fig. 1A). These basic residues are expected to play a major role in assembly as a result of their interaction with the negatively charged viral RNA and the requirement to neutralize that charge within virions for structural stability (Vriend *et al.*, 1986). Previously, we have observed that CP deletion mutants lacking the N-terminal 15 residues are defective in systemic infection, and deletions of either the first 10 residues or sets of five contiguous residues between residues 21 and 50 produce a substantial reduction in virion yield (S.-H. Park *et al.*, unpublished data). To elucidate any additional effects of these basic residues on the RCNMV infection process, we introduced nine alanine mutations into an infectious RNA-1 construct and co-inoculated them with WT RNA-2 into *N. benthamiana*.

One interesting, albeit not totally unexpected, finding from this study was that CP LRM mutations altered host symptomatology (Figs 2 and 3). It has been reported that CPs of several viruses play a role in symptom expression (Banerjee *et al.*, 1995; Bol, 2005; Culver and Dawson, 1989; Knorr and Dawson, 1988; Lin and Heaton, 1999; Neeleman *et al.*, 1991; Saito *et al.*, 1989; Shintaku *et al.*, 1992). Minor modifications in the CP, such as single amino acid substitutions, can result in a significant alteration of symptoms. For instance, mutational analysis of the *Cowpea chlorotic mottle virus* (CCMV) CP showed that residue 151 determined the symptom phenotype of strains CCMV-T and CCMV-M on cowpea (de Assis Filho *et al.*, 2002). Furthermore, two TCV CP mutants (TCV-M22 and TCV-M33) showed cell-to-cell and systemic movement similar to WT TCV, but milder systemic symptoms than for WT TCV on *N. benthamiana* (Lin and Heaton, 1999). Some plant viral CPs act as elicitors in specific hosts, causing an HR, which leads to necrosis and cell death at sites of infection to prevent spread of the pathogen (Bol *et al.*, 1990). Point mutations in the CP of *Tobacco mosaic virus* altered the symptomatology from a WT mosaic to an HR in *N. sylvestris* containing the N' resistance gene (Culver and Dawson, 1989; Saito *et al.*, 1989). The CPs of *Paprika mild mottle virus* and *Pepper mild mottle virus* also act as elicitors of the HR in *Capsicum* containing either the L² or L⁴ resistance genes (Gilardi *et al.*, 2004).

Despite the many studies showing that CP mutations can alter symptomatology, there appears to be no general correlation between a particular CP domain and the symptoms produced. In addition, there are numerous other virus–host interactions, as well as environmental factors, ultimately involved in disease induction and symptom expression. Interestingly, the symptomatology of RCNMV mutants K4A and K7A/K8A (severe necrosis on inoculated leaves) was comparable with that produced by an HR-like

Fig. 5 The effects of alanine substitution mutants (ASMs) on encapsidation. (A, C) Ethidium bromide (EtBr) staining of virions purified from inoculated (A) and noninoculated (C) leaves electrophoresed through native 1% agarose/TAE gels. (B, D) The same gels as in (A) and (C) were stained with Coomassie brilliant blue R250 for virion proteins from inoculated (B) and noninoculated (D) leaves. (E–H) Western blot of purified virions (E, F) and total protein extracts (G, H). Virion and total protein samples were prepared from sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, denatured by boiling and electrophoresed through a 12% polyacrylamide gel. The loaded sample volume of virions was increased 20-fold in (F) relative to (E). WT, wild-type.

response. The CP RNA coding region (not the CP itself) of the closely related *Cymbidium ringspot virus* (CymRSV) has been shown to be an elicitor of HR on *Datura stramonium* (Szittyá and Burgyn, 2001). This raises the possibility that the RCNMV CP is an effector blocking pathogen-associated molecular pattern-triggered immunity (PTI) and, when the CP is present, it is respon-

sible for limiting the host response to infection to allow systemic spread. It is unlikely that virion assembly *per se* produces this phenotype, as both K4A and K7A/K8A are assembly competent, yet display severe necrosis. More likely, the CP may interact, either directly or indirectly, with unknown host factors involved in the HR. The RCNMV CP mutants, K4A and K7A/K8A, induced a local

Fig. 6 Analysis of virions produced by alanine substitution mutants (ASMs). (A) Transmission electron micrographs of ASMs. Virions were purified from noninoculated leaves of plants inoculated with the mutants indicated in each panel. Panel depicting mutants K25A, K33A and K38A is representative of the results obtained individually with each mutant. (B, C) Northern blot analysis of viral RNA isolated from purified virions of inoculated (B) and noninoculated (C) leaves. Viral RNAs were analysed by 1% agarose gel and probed with a combination of RNA-1- and RNA-2-specific probes. Samples are as follows: TSC, *in vitro*-transcribed RNA; lane 1, wild-type (WT); lane 2, K4A; lane 3, K7A/K8A; lane 4, K10A/R12A; lane 5, R16A/R18A; lane 6, K25A; lane 7, K33A; lane 8, K38A; lane 9, K45A; lane 10, R48A.

and systemic HR, which indicates that infection is restricted to a lesion, but not prevented from systemic infection. It is unlikely that the symptomatology changes are caused by effects on the host RNA silencing mechanism, as previous reports have implicated either the virally encoded replication proteins (p27 and p88) in an actively replicating complex (Takeda *et al.*, 2005) or the MP (Powers *et al.*, 2008) as the RCNMV encoded suppressors of RNA silencing. Our results, however, obviously demonstrate that the N-terminal LRM of the CP is involved in symptom modulation, even though the molecular mechanism remains undetermined. The potential interactions between the RCNMV CP and host factor(s) leading to symptom alteration should be addressed in future studies.

The other major conclusion from our results is that RCNMV is able to systemically infect *N. benthamiana* without virion production. In the present study, point mutants K25A, K33A and K38A did not produce detectable virions when assayed by either TEM (Fig. 6) or agarose gel electrophoresis (Fig. 5) from inoculated or upper noninoculated leaves. The absence of CP expression (the CP-null mutant) also allowed systemic movement in a nonvirion form (Fig. S1). Although an earlier study suggested that virion formation was required for RCNMV long-distance movement (Vaewhongs and Lommel, 1995), those experiments involved RNA-1-only inoculations onto transgenic *N. benthamiana* expressing the RCNMV MP, without RNA-2, which is necessary for both the induction of CP expression and virion assembly (Basnayake

et al., 2009; Sit *et al.*, 1998). Recently, we have reported that N-terminal deletions greater than 10 residues abolish virion formation and systemic movement, and have suggested that the RNA region spanning CP residues 5–15 may contain an element involved in replication and that the removal of this element may lead to decreased accumulation levels, resulting in reduced or impaired systemic movement (S.-H. Park *et al.*, unpublished data). Although systemic movement without virion formation was also observed in a previous study involving CP deletion mutants (Xiong *et al.*, 1993), those findings involved plants incubated at a relatively low temperature (15 °C versus 25 °C in the present study) over a much longer time frame (28 days versus 7 days in the present study). The requirement for a longer incubation period in the previous study may have been caused by the experimental design, in which noninoculated leaves were not assayed unless a specific symptomatology developed (oak-leaf necrosis), as well as the utilization of a less infectious RNA-1 construct (pRC1IG; Xiong and Lommel, 1991) as the basis for mutant constructs. As mentioned above, the CP ORF deletions may have unintentionally altered viral replication levels by removing/altering critical RNA replication elements. Moreover, virion formation is not a requirement of systemic infection in *N. benthamiana* for the well-characterized tombusviruses TBSV, CymRSV and *Cucumber necrosis virus* (Dalmay *et al.*, 1992; McLean *et al.*, 1993; Scholthof *et al.*, 1996).

Although virion accumulation was not absolutely necessary for systemic movement, it appears that CP production, even at severely reduced levels, aids in systemic infection. The analysis of RNAs and CP extracted from purified virions of mutants K25A, K33A and K38A revealed highly reduced levels of each component even when substantial amounts of sample were examined (Figs 5E, 5F, 6B and 6C). This significant reduction in virion yields suggests that these residues are crucial for stable virion formation and that their alteration might disturb RNA binding within virions and probably lead to unstable virions. Under this scenario, we can envisage that virions might not be detected because of degradation or aggregation during sample preparation. These results also suggest that there may be other CP–RNA complexes in addition to virions which were obtained through our virion extraction method. Accordingly, the long-distance transport of RCNMV viral RNA through *N. benthamiana* might occur via an alternative physical form, such as a viral ribonucleoprotein complex or some other nonvirion form minus CP. The remarkable susceptibility of *N. benthamiana* to a diverse array of plant viruses might be one explanation for the feasibility of long-distance transport involving nonvirion complexes. However, virions appear to be favoured for the rapid, long-distance spread of RCNMV.

Lastly, we were intrigued by the inverse correlation between symptom severity and viral RNA accumulation in noninoculated leaves. This was most striking for mutants K33A and K38A, which appeared to be much milder than WT RCNMV, yet accumulated

viral RNA to a much higher level. As mentioned above, the RCNMV CP might be an effector of PTI or an elicitor of the HR. Thus, when the CP is unable to induce either an HR or a PTI, the viral RNAs may accumulate to a higher level in the upper leaves, as they are not primed for basal defence. In the case of mutant K25A, it is clear that this mutant must still be able to induce some basal defence, as it accumulates less than mutants K33A and K38A, which do not. However, all three of these mutants do not induce normal disease symptoms, which may relate to another property of the CP. As few or no stable virions are formed by these mutants, they may all generate CPs that are insoluble and do not induce symptoms. By contrast, mutants K4A and K7A/K8A elicited severe necrotic symptoms, systemic HR and their viral genomic RNAs in systemic leaves accumulated to lower levels than that of WT RCNMV. Less viral RNA accumulation of CP mutants K4A and K7A/K8A in the noninoculated leaves could be caused by a systemic acquired resistance induced by local necrosis, which is sufficient to reduce viral RNA accumulation, but does not prevent further infection. Alternatively, the severe or milder symptoms may be the result of mutants having altered affinities for host factors which may normally respond to viral RNA accumulation, thus negating the usual and customary response to such an infection. It would be interesting to examine host gene expression profiles for possible changes elicited by the various CP mutants versus a WT RCNMV infection. Another interesting observation is that the ratio of RNA-1 to RNA-2 is altered in some mutants, suggesting a role for these residues in packaging discrimination between RNA-1 and RNA-2. We are currently investigating this observation further.

In summary, our findings demonstrate that the multifunctional nature of the RCNMV CP can be revealed by mutagenesis to the LRM. These mutations show that the CP is involved not only with regard to its primary structural role in virion formation, but also in symptom development, viral RNA accumulation and systemic movement within *N. benthamiana*.

EXPERIMENTAL PROCEDURES

Plasmid constructs

Site-directed mutagenesis of positively charged amino acids in the RCNMV CP LRM was performed on the RCNMV RNA-1 infectious clone pRC169 (Sit *et al.*, 1998) via PCR using the megaprimer method (Barik, 1993) with specified primer pairs (Table S1, see Supporting Information). All amplified products were digested with *Bgl*II and *Mlu*I restriction enzymes and cloned back into pRC169. The resulting constructs were sequenced to confirm the insertion of the correct mutations.

Plant inoculation

Plasmids pRC169, RNA-1 ASMs and pRC2-IG (WT RNA-2 construct) were linearized with *Sma*I prior to *in vitro* T7 RNA transcription, as described

previously (Sit *et al.*, 1998). Five microlitres of each genomic RNA transcript were mixed with 110 μ L of 5 mM sodium phosphate buffer, pH 7.0, prior to inoculation onto two carborundum-dusted leaves of each independent *N. benthamiana* plant at the six- to eight-leaf stage. Plants were maintained at 25 °C in a growth chamber with a 16-h photoperiod.

Total leaf RNA and protein extraction

Leaves of *N. benthamiana* plants were harvested at 4 dpi (inoculated and upper noninoculated leaves) and 7 dpi (upper noninoculated leaves only). Total RNA was isolated from harvested leaf samples (0.1 and 0.5 g) with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Total leaf proteins were prepared from harvested leaf samples (0.5 g) by grinding in an equal volume (1 : 1 m/v) of 2 × Laemmli buffer, followed by heating at 100 °C for 3 min and chilling on ice for 5 min.

Virion purification and viral RNA extraction

Virions were prepared by homogenizing 0.5 g of infected leaf tissue in 1 mL of 0.2 M sodium acetate, pH 5.2, containing 0.1% β -mercaptoethanol, as described previously (Basnayake *et al.*, 2009). After incubation on ice for 10 min, the homogenate was centrifuged at 15 110 *g* for 10 min. The supernatant was filtered through Miracloth and one-quarter volume of 40% polyethylene glycol (PEG) 8000/1 M NaCl was added, followed by incubation on ice for 30 min. Virions were collected by centrifugation at 15 110 *g* for 10 min and resuspended in 50 μ L of 10 mM Tris-HCl, pH 6.5, at 4 °C overnight. Twenty microlitres of virions were electrophoresed through 1% agarose gels in 0.5 × TAE buffer [20 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and bands were detected by staining with either ethidium bromide or CBB-R250.

Viral RNA was extracted from purified virion preparations with phenol-chloroform in the presence of 2% sodium dodecylsulphate (SDS), as described previously (Lommel *et al.*, 1988). After ethanol precipitation, the collected RNA was resuspended in 10 μ L of nuclease-free water.

Electron microscopy

Purified virions were absorbed to Formvar-coated copper grids and negatively stained with 2% phosphotungstic acid. Electron micrographs of virions were obtained using a JEOL-5410 LV transmission electron microscope (Olympus, Tokyo, Japan) at 200 000× magnification.

Western blotting

Purified virions and total protein extracts were dissolved in 2 × Laemmli buffer (Laemmli, 1970) and heated at 100 °C for 3 min, followed by chilling on ice for 5 min. Samples were separated on a 12% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride membrane in Towbin buffer with 20% methanol using a Bio-Rad Trans-Blot® SD transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% skimmed milk in 1 × TBST (20 mM Tris-HCl, 500 mM NaCl, 0.1% Tween-20, pH 7.5) at 4 °C overnight, followed by incubation with anti-RCNMV CP

rabbit polyclonal antisera diluted 1 : 1000 with 5% skimmed milk in 1 × TBST at room temperature for 1 h. The membrane was washed three times in 1 × TBST, followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated antisera (Sigma, St. Louis, MO, USA) at room temperature for 1 h. Detection was performed using the Amersham ECL™ Advance Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions, with protein bands being captured on X-ray film (Agfa-Gevaert, Mortsel, Belgium).

Northern blot analysis

Northern blot hybridization analysis was performed using the Northern Max-Gly kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Total RNAs were denatured at 50 °C in glyoxal–dimethylsulphoxide sample buffer for 30 min and fractionated in a 1% low electroendosmosis-agarose gel (Ambion). Fractionated RNA was transferred to a BrightStar-Plus membrane (Ambion) by the downward capillary transfer method. Transferred RNA was cross-linked to the membrane with UV light and the membrane was processed for prehybridization and hybridization in ULTRAhyb® buffer (Ambion) at 42 °C. ³²P-Labelled DNA probes corresponding to the 3' region of RNA-1 and RNA-2 were prepared as described previously (Mizumoto *et al.*, 2002). The hybridized membranes were exposed to BAS imaging plates (Fuji Photo Film, Tokyo, Japan) and visualized using a Fujix BAS 2500 bioimage analyser (Fuji Photo Film).

qRT-PCR

Total RNAs extracted from inoculated and noninoculated leaves (described above) were treated with TURBO DNA-free™ (Ambion) to remove contaminating genomic DNA. One microgram of total RNA was reverse transcribed using M-MuLV reverse transcriptase (Promega, Madison, WI, USA) and either an RCNMV-specific 3' primer (3'-p27-115 for RNA-1 and 3'-R2-108 for RNA-2) or oligo (dT) 3' primer. The expression levels of genomic RNAs were measured by qRT-PCR with a desired combination of specific primers (5'-p27-115 and 3'-p27-115 for RNA-1 and 5'-R2-108 and 3'-R2-108 for RNA-2; Table S1) and the CFX96™ Real-Time PCR System (Bio-Rad) using SsoFast™ EvaGreen® Supermix (Bio-Rad), according to the manufacturer's instructions. The thermal profile was 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 5 s. An endogenous gene, *ubiquitin 3* (*Ubi3*), was used for the normalization of different RNA quantities (Rotenberg *et al.*, 2006).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Symptom development and viral RNA accumulation of a coat protein (CP)-null mutant. (A) Symptoms of *Nicotiana benthamiana* plants and leaves inoculated with wild-type (WT) RCNMV and the CP-null mutant. Photographs were taken at 14 days post-inoculation (dpi). (B) Viral RNA accumulation in inoculated (white bars) and upper noninoculated (grey bars) leaves of *N. benthamiana*. Virus RNA accumulation was measured by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). Total RNA was extracted from inoculated and upper noninoculated leaves at 4 and 7 dpi, respectively. Each value is the mean and standard error relative to the WT compiled from at least three independent experiments for both RNA-1 and RNA-2 accumulation.

Table S1 Oligonucleotides used in this study.

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