

# Maintenance of coat protein N-terminal net charge and not primary sequence is essential for zucchini yellow mosaic virus systemic infectivity

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*Zucchini yellow mosaic virus* (ZYMV) surface exposed coat protein (CP) N-terminal domain (Nt) is 43 aa long and contains an equal number of positively and negatively charged amino acid residues (CP-Nt net charge = 0). A ZYMV-AGII truncation mutant lacking the first 20 aa of its CP-Nt (AGII-CP $\Delta$ 20; CP-Nt net charge = +2) was found to be systemically non-infectious even though AGII mutants harbouring larger CP-Nt deletions were previously demonstrated to be fully infectious. Nevertheless, AGII-CP $\Delta$ 20 infectivity was restored by fusion to its CP-Nt two Asp residues or a negatively charged Myc peptide, both predicted to neutralize CP-Nt net positive charge. To evaluate further the significance of CP-Nt net charge for AGII infectivity, a series of CP-Nt net charge mutants was generated and analysed for systemic infectivity of squash plants. AGII-CP<sup>KKK</sup> harbouring a CP-Nt amino fusion of three Lys residues (CP-Nt net charge = +3) was not systemically infectious. Addition of up to four Asp residues to CP-Nt did not abolish virus infectivity, although certain mutants were genetically unstable and had delayed infectivity. Addition of five negatively charged residues abolished infectivity (AGII-CP<sup>DDDDD</sup>; CP-Nt net charge = -5) even though a recombinant CP<sup>DDDDD</sup> could assemble into potyviral-like particle in bacteria. Neutralization of CP-Nt net charge by fusing Asp or Lys residues recovered infectivity of AGII-CP<sup>KKK</sup> and AGII-CP<sup>DDDDD</sup>. GFP-tagging of these mutants has demonstrated that both viruses have defective cell-to-cell movement. Together, these findings suggest that maintenance of CP-Nt net charge and not primary sequence is essential for ZYMV infectivity.

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## INTRODUCTION

Potviruses comprise the largest group of viruses that collectively infect most crop plants and many other wild plant species. Potviruses have flexuous filamentous particles 700–900 nm long and 10–15 nm in diameter. Their genomes are single-strand, positive-sense RNA molecules of approximately 10 000 nt, encapsidated by nearly 2000 subunits of a single type of coat protein (CP) (Shukla *et al.*, 1994). Immunological, biochemical and structural studies of this protein have demonstrated structural features similar to the CP of *Tobacco mosaic virus* (TMV) (McLachlan *et al.*, 1980) and *Potato virus X* (Sawyer *et al.*, 1987). Like those proteins, potyviral CP is a three-domain protein with N- and C-terminal regions exposed on the virion surface (Allison *et al.*, 1985; Baratova *et al.*, 2001; Shukla *et al.*, 1988) and a conserved core domain that was shown to be required for virus assembly (Dolja *et al.*, 1995; Jagadish *et al.*, 1991;

Varrelmann & Maiss, 2000), plasmodesmatal gating (Rojas *et al.*, 1997) and cell-to-cell movement (Dolja *et al.*, 1995).

In contrast to the conserved core, the exposed CP N-terminal domain (CP-Nt) is not conserved in sequence and varies considerably in length (Shukla *et al.*, 1989; Shukla & Ward, 1989). It has been proposed that this broad variability represents a way for the virus to interact with specific host factors for movement and perhaps other functions in the virus life-cycle (Urququi-Inchima *et al.*, 2001); and it has been demonstrated that CP-Nt assists in aphid transmission via its conserved DAG motif (Atreya *et al.*, 1991), through interaction with the virus encoded helper component proteinase (Blanc *et al.*, 1997; Peng *et al.*, 1998). It was shown that the removal of CP-Nt by limited trypsin proteolysis did not affect particle morphology, suggesting that this domain might not be involved in particle assembly (Jagadish *et al.*, 1993). Furthermore, recently, it was demonstrated that a deletion of up to 112 aa from the CP N-terminal of *Tobacco etch virus* (TEV), including its complete CP-Nt, did not abolish its ability to assemble into potyviral-like particles (PVLPS) in bacteria (Voloudakis

Supplementary table presenting CP-Nt net charge of various potviruses is available in JGV Online.

*et al.*, 2004). In contrast, a complete deletion of *Pepper vein banding virus* CP-Nt prevented the truncated recombinant CP to assemble into PVLPS in bacteria suggesting a role for CP-Nt in the initiation of particle assembly (Anindya & Savithri, 2003). A number of studies have shown that CP-Nt is involved in virus long-distance movement and systemic spread. TEV mutants with deletions in the CP N- or C-terminal domains have produced virions *in vivo* but the virus was defective in long-distance movement *in planta* (Dolja *et al.*, 1994, 1995). Mutational analysis demonstrated that changes of Ser47 to Pro of the *Pea seed-borne mosaic virus* CP (Andersen & Johansen, 1998) can modulate the ability of the virus to move systemically in *Chenopodium quinoa*. Also, substitution of Asp to Lys in the DAG motif of the *Tobacco vein mottling virus* (TVMV) and TEV CP-Nts abolished systemic movement (Lopez-Moya & Pirone, 1998) concluding that charge changes made specifically in the DAG motif alter virus capacity for systemic movement (Lopez-Moya & Pirone, 1998).

ZYMV CP-Nt is 43–45 aa long with its putative trypsin protease motif presumed to be positioned between amino acids Lys42 and Asp43, located in the KDKD motif (Shukla *et al.*, 1988). We have previously demonstrated that foreign peptides of up to 31 aa long can substitute for part or all of ZYMV CP-Nt domain while maintaining systemic infectivity, suggesting that ZYMV CP-Nt per se is not essential for assembly or movement (Arazi *et al.*, 2001a). Nevertheless, in several cases fusion of positively charged peptides to CP-Nt failed to result in an infectious cDNA clone even though fused peptides were shorter than 31 aa (Arazi *et al.*, unpublished results). These observations were reminiscent of results shown for TMV (Bendahmane *et al.*, 1999) and *Cowpea mosaic virus* (CPMV) (Porta *et al.*, 2003) where fusion of positively charged peptides to exposed parts of their CP affected their systemic movement.

In this study, we investigated the effect of ZYMV CP-Nt charge on virus systemic infection. We generated a series of virus mutants, each harbouring a CP-Nt with modified net charge, and analysed the effect of each charge change on virus infectivity. This analysis revealed a correlation between CP-Nt net charge and virus systemic infectivity, suggesting that maintenance of CP-Nt net charge and not primary sequence is necessary for ZYMV infectivity.

## METHODS

**Plasmid construction.** To enable one-step cloning of foreign sequences into the attenuated ZYMV-AGII virus vector, we have eliminated two *Pst*I sites found at positions 2228 and 3830 of the viral cDNA (GenBank accession no. AY188994) by site-directed mutagenesis to generate a modified AGII, which contains one unique *Pst*I site, inserted previously as part of an artificial polylinker cloned upstream of the CP encoding sequence (Arazi *et al.*, 2001b). Modified ZYMV-AGII showed identical symptoms and infectivity kinetics as parental AGII and hence will be referred here as AGII. The coding region of AGII, including 35S promoter and NOS terminator, was then digested by *Not*I/*Kpn*I and subcloned into the corresponding sites of a pGreen plasmid (Hellens *et al.*, 2000).

Foreign amino acid residues were inserted upstream of AGII CP coding sequence by PCR mutagenesis, with AGII cDNA as a template. Sense primers contained a *Pst*I site at their 5' end, followed by the sequence encoding foreign amino acid residues and a homologous CP sequence. The CP homologous antisense primer (5'-CATTTCCTTTTCACGCGTGGC-3') spans a unique *Mlu*I site found 205 nt downstream the CP coding sequence. The amplified fragments were double digested by *Pst*I/*Mlu*I and cloned into *Pst*I/*Mlu*I digested AGII cDNA clone.

For bacterial expression, the coding region of wild-type CP and mutant CPs with N-terminal fusion of three Lys (CP<sup>KKK</sup>) or five Asp (CP<sup>DDDD</sup>) residues were amplified by PCR with AGII cDNA as a template. Sense and antisense (5'-CCGCTCGAGTTACTGCATTG-TATTCACCTAG-3') primers contained a *Bsp*HI and a *Xho*I site (underlined) at their 5' end, respectively, were used. Amplified PCR fragments were digested with *Bsp*HI and *Xho*I and ligated into *Nco*I/*Xho*I sites of pET28a(+) expression vector (Novagen) generating pET28a-CP, pET28a-CP<sup>KKK</sup> and pET28a-CP<sup>DDDD</sup> with an additional methionine at the 5' end of each CP to enable expression in bacteria.

For AGII-GFP expression, firstly, GFP S65T coding region (Reichel *et al.*, 1996) was amplified by PCR. Sense and antisense (5'-CGC-GTGCACACTGTAATGCTGCAGTCA-3') primers contained a *Pst*I and a *Sal*I (underlined) site at their 5' end, respectively. Amplified PCR fragment was cloned into *Pst*I/*Sal*I sites of AGII cDNA to generate AGII-GFP. Foreign amino acid residues were then inserted upstream of AGII-GFP CP coding sequence using the same strategy as described for AGII mutants except that the sense primer contained *Sal*I instead of *Pst*I.

**Plant growth, inoculation and symptom evaluation.** Zucchini squash (*Cucurbita pepo* L. cv. zucchini) plants were grown in a growth chamber under continuous light at 23 °C. Seedlings were selected for experimental use when their cotyledons were fully expanded. Particle bombardment inoculation of AGII and various mutants was performed with a hand-held device, the handgun (Gal-On *et al.*, 1997). After bombardment inoculation, squash seedlings were grown and examined daily for symptom development, and the first appearance of symptoms on non-inoculated leaves was recorded.

**Detection of viral RNA and proteins.** Total RNA (2–5 µg) was extracted from three leaf disks (one per leaf; 9 mm diameter), collected from non-inoculated leaves of an infected plant, using TRI-reagent (Sigma). RT-PCR of virus progeny was conducted in a one-tube, single-step method with CP-Nt flanking primers 5'-AGCTCCATACATAGCTGAGACA-3' and 5'-TGGTTGAACCA-AGAGGCGAA-3' as described by Arazi *et al.* (2001b). Resulting amplified fragments were sequenced directly.

Total proteins were extracted separately from three squash seedlings. Samples (~70 mg; six leaf disks, two of each plant) from non-inoculated leaves were collected. Each sample was ground in 150 µl ESB buffer [75 mM Tris/HCl pH 6.8, 9 M urea, 4.5% (v/v) SDS, 7.5% (v/v) β-mercaptoethanol], boiled for 5 min and cooled on ice. Cooled homogenates were centrifuged for 10 min at 10 000 g, and 100 µl supernatant containing total leaf proteins was mixed with 100 µl 2 × SDS-PAGE loading buffer. A 10–15-µl sample of the mixture was fractionated by SDS-PAGE on a 12.5% polyacrylamide gel. The fractionated proteins were electroblotted onto nitrocellulose membranes and probed with a monoclonal antiserum specific to ZYMV CP-Nt (AB6; 1:500) (Desbiez *et al.*, 1997) or an anti-CP polyclonal antibody (1:2000).

**Expression of recombinant CP and evaluation of potyvirus-like particle (PVL) formation by electron microscopy.** Cultures of *E. coli* BL21 Rosetta (Novagen) transformed with

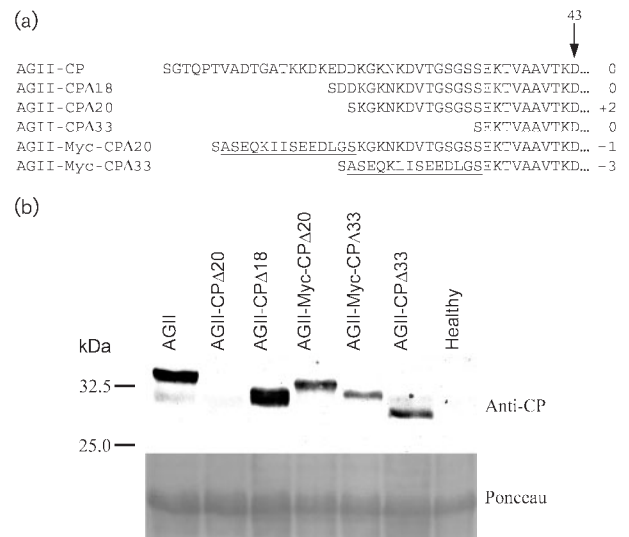
pET28a-CP, pET28a-CP<sup>KKK</sup>, pET28a-CP<sup>DDDDD</sup> and pET28a, were grown overnight at 37 °C. One ml of each culture was diluted 1:100 in fresh medium, grown to OD value of 0.5 at 28 °C, induced by the addition of 200 µM IPTG (final concentration) and further incubated at 28 °C overnight. Bacterial cells were then pelleted and resuspended in 5 ml extraction buffer (50 mM Tris/HCl pH 7.6, 2 mM EDTA, 1 mM DTT, 1 mM PMSF). Lysozyme was added to a final concentration of 200 µg ml<sup>-1</sup> and the reaction mixture was incubated for 10 min at room temperature followed by 15 min on ice until the bacterial cells lysed. Digestion of bacterial genomic DNA was done by addition of DNase I (Sigma) to a final concentration of 50 µg ml<sup>-1</sup> together with 3 mM MgCl<sub>2</sub> and incubation at room temperature for 10 min. Bacterial extract was then centrifuged for 30 min at 14 000 r.p.m. (Beckman SS34 rotor) and the supernatant fraction was collected. The presence of PVLPs in the bacterial extract was verified by visualization under the electron microscope. Formvar carbon-coated grids were incubated on droplets of supernatants at room temperature for 1 min, washed three times with water and negatively stained with 2% uranyl acetate. Images were taken on a JEOL JEM-100CXII electron microscope.

**Visualization of GFP fluorescence.** AGII, AGII-GFP, AGII-GFP-CP<sup>KKK</sup> and AGII-GFP-CP<sup>DDDDD</sup> cDNAs were bombarded into detached cotyledon epidermal cells of squash, under vacuum conditions, using a particle bombardment gun (Gray *et al.*, 1994). Bombarded cotyledons were placed on 1% agarose in closed Petri dish and maintained in a growth chamber under 16 h light at 26 °C. Images were acquired using a confocal laser-scanning microscope system (Olympus IX81) equipped with an argon laser. The GFP images were obtained at an excitation wavelength of 488 nm and a 515–525 nm emission filter. Transmitted light images were acquired using Nomarski differential interference contrast.

## RESULTS

### A non-infectious AGII mutant becomes infectious upon fusion of negatively charged residues to its truncated CP-Nt

We have confirmed that an AGII mutant harbouring an N-terminal deletion of 33 aa from its CP (AGII-CPΔ33; Fig. 1a) is infectious and spreads systemically in squash (Table 1 and Fig. 1b), demonstrating that at least the first 33 aa residues of CP-Nt are dispensable for virus assembly and systemic movement. Surprisingly, a deletion of only 20 aa from the N-terminal of CP (AGII-CPΔ20; Fig. 1a) resulted in a non-infectious cDNA clone (Table 1 and Fig. 1b). Interestingly, systemic infectivity of AGII-CPΔ20 was recovered by addition of either two native Asp residues, upstream of Lys21 (AGII-CPΔ18; Fig. 1a) or a 14 aa foreign Myc peptide (AGII-Myc-CPΔ20; Fig. 1a). AGII-CPΔ18 and AGII-CP-MycΔ20 viruses were systemically infectious (Table 1) and symptoms appeared 7–8 days post-inoculation (p.i.) with characteristics similar to those of parental attenuated AGII virus (Table 1). Immunoblotting of systemically infected squash leaves with anti ZYMV CP antibody detected a specific band in AGII-CPΔ18 and AGII-CP-MycΔ20 extracts but not in the non-infectious AGII-CPΔ20 (Fig. 1b). In addition, mutant viruses were genetically stable in plants as demonstrated by RT-PCR analysis and direct sequencing of the amplified product (Table 1).



**Fig. 1.** CP accumulation of AGII CP-Nt truncated mutants in squash. (a) CP-Nt amino acid sequence of different truncated mutants. The position of predicted last residue in CP-Nt is indicated by an arrow. Residues encoding the Myc peptide are underlined. CP-Nt predicted net charge for each mutant is indicated on the right. (b) Immunoblot analysis of squash inoculated with AGII CP-Nt truncation mutants shown in (a) 14 days p.i. Total extracts (15 µl) of systemic squash leaves were separated by SDS-PAGE (12.5%), blotted and probed with anti-CP polyclonal antibody. Relative loading of protein in each lane is shown by Ponceau staining. Extracts from non-inoculated (Healthy) plants were used as a negative control. The positions of molecular-mass standards (kDa) are indicated on the left.

We addressed the basis for AGII-CPΔ20 recovery of infectivity by comparing the composition of amino acids in CPΔ20 Nt versus wild-type and the reversion mutants CP-Nts. We found that wild-type CP-Nt contains an equal number of positively (eight Lys) and negatively (six Asp and two Glu) charged amino acid residues giving a net charge of 0 (Fig. 1a). The truncated CPΔ20 Nt contains five Lys residues versus three negatively charged residue giving a net charge of +2 (Fig. 1a). Like the wild-type CP, CPΔ18 and CPΔ33 Nts contain an equal number of positively and negatively charged residues (Fig. 1a). CP-MycΔ20 Nt contains one more negatively than positively charged residues giving a net charge of -1 (Fig. 1a). This analysis raised the possibility that the excess net positive charge, found only in CPΔ20 Nt, might disrupt systemic infectivity.

### A CP-Nt net positive charge larger than +1 prevents virus systemic infectivity

To validate our hypothesis, we constructed a series of mutant AGII cDNA clones designed to express versions of full-length CP-Nt with increased net positive charge, by N-terminal fusion of either Lys or Arg residues (Fig. 2a

**Table 1.** Characterization of AGII positively charged and truncated CP-Nt mutants

Clone	CP-Nt pI/net charge*	Systemic infectivity (%)†	Time of symptom appearance (days p.i.)	Progeny CP-Nt sequence‡	Progeny CP-Nt pI/net charge*
AGII-CP	6·09/0	100	6–7	SGTQ	6·09/0
AGII-CPΔ18	6·00/0	100	7–8	SDDKGGK	6·00/0
AGII-CPΔ20	9·40/+2	0	–	–	–
AGII-CPΔ33	5·91/0	100	7	SEKTVA	5·91/0
AGII-Myc-CPΔ20	5·18/–1	100	7–8	<u>SASEQK...K</u> GGK	5·18/–1
AGII-CP <sup>K</sup>	7·98/+1	100	7	SKGTQ	7·98/+1
AGII-CP <sup>R</sup>	8·00/+1	100	7	SRGTQ	8·00/+1
AGII-CP <sup>KK</sup>	9·05/+2	30/NO	–	SEKGTQ	6·66/0
				SKEGTQ	6·66/0
AGII-CP <sup>RR</sup>	9·16/+2	28/NO	–	SRCGTQ	7·82/+1
				SRHGTQ	8·03/+1
				SQRGTQ	8·00/+1
AGII-CP <sup>KKK</sup>	9·35/+3	0	–	–	–
AGII-CP <sup>AAA</sup>	6·09/0	100	6–7	SAAAGTQ	6·09/0
AGII-CP <sup>KKKAA</sup>	9·35/+3	0	–	–	–
AGII-CP <sup>KKKDD</sup>	7·93/+1	100	7	SKKKDDGTQ	7·93/+1
AGII-CP <sup>DDKKK</sup>	7·93/+1	100	7	SDDKKKGTQ	7·93/+1

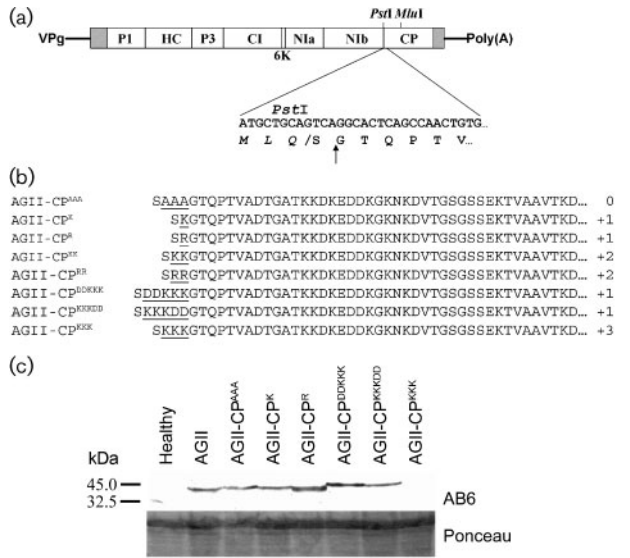
\*Predicated isoelectric point (pI) was determined using ExPASy ProtParam tool (<http://au.expasy.org/tools/protparam.html>). Net charge = total number of positively charged residues (Lys and/or Arg) minus total number of negatively charged residues (Glu and/or Asp).

†Systemic infectivity of indicated clone on squash plants (at least nine plants tested). 0, no virus accumulation and visible symptoms were observed. NO, RT-PCR and sequencing analysis could not recover the original clone sequence.

‡As determined by RT-PCR of virus progeny and direct sequencing of amplified product. Bold residues are those differing from originally inoculated constructs. Residues encoding Myc peptide are underlined.

and b), and tested the systemic infectivity of the mutant cDNAs. Squash seedlings were inoculated by particle bombardment with various mutant cDNA clones. Symptom appearance on non-inoculated leaves, indicative of systemic spread, was recorded (Table 1) and the presence of virions was verified under the electron microscope after partial purification (data not shown). RT-PCR of virus progeny and direct sequencing of the amplified product was done in each case to confirm the authenticity and genetic stability of each mutant (Table 1). Virus accumulation of genetically stable mutants was estimated by Western blot analysis 14 days p.i. with anti-CP AB6 monoclonal antibody (Desbiez *et al.*, 1997). Modification of CP-Nt by the addition of one positively charged Lys (AGII-CP<sup>K</sup>) or Arg (AGII-CP<sup>R</sup>) residue did not compromise virus systemic infectivity and CP accumulation level (Table 1 and Fig. 2c). However, modification of CP-Nt by addition of two Lys (AGII-CP<sup>KK</sup>) or Arg (AGII-CP<sup>RR</sup>) residues (Fig. 2b) resulted in non-infectious clones that sometimes caused a delayed systemic infection (Table 1). RT-PCR and sequencing analysis of progeny viruses from infected plants did not recover the original clone sequence. Instead, a change of one of the added Lys to an acidic Glu or one of the added Arg to a weakly charged amino acid residue (Cys, His or Gln) was found (Table 1). All the changes occurred by means of a single nucleotide change *in planta*. These changes, which spontaneously eliminated one positively

charged residue, suggest that a CP-Nt net charge of +1 is tolerable by the virus, as was the case with infectious AGII-CP<sup>K</sup> and AGII-CP<sup>R</sup> (Table 1). Fusion of three Lys residues to CP-Nt (AGII-CP<sup>KKK</sup>) resulted in a non-infectious clone and late systemic infection was not recorded (Table 1). In addition, no accumulation of CP<sup>KKK</sup> could be detected by Western blot analysis (Fig. 2c). To test whether AGII-CP<sup>KKK</sup> lack of infectivity is directly correlated to the specific fusion of three basic residues to CP-Nt, we fused three neutral Ala residues at a similar location to generate AGII-CP<sup>AAA</sup>, which harbours a CP-Nt net charge of 0 like the wild-type CP-Nt. In contrast to AGII-CP<sup>KKK</sup>, AGII-CP<sup>AAA</sup> was systemically infectious and accumulated to a similar level as parental AGII (Table 1 and Fig. 2c). To confirm that CP-Nt net positive charge added in AGII-CP<sup>KKK</sup> is the cause for non-infectivity, we generated mutants where added positive charge was partially neutralized by the addition of two negatively charged Asp residues either upstream (AGII-CP<sup>DDKKK</sup>) or downstream (AGII-CP<sup>KKKDD</sup>) of the KKK residues (Fig. 2b). These additions changed the CP-Nt net charge from +3 to +1, which is predicted to support systemic infectivity as was demonstrated with AGII-CP<sup>K</sup> and AGII-CP<sup>R</sup> (Table 1). Indeed, AGII-CP<sup>DDKKK</sup> and AGII-CP<sup>KKKDD</sup> were systemically infectious 7 days p.i., accumulated to a similar level as parental AGII (Fig. 2c) and were genetically stable (Table 1). A control clone

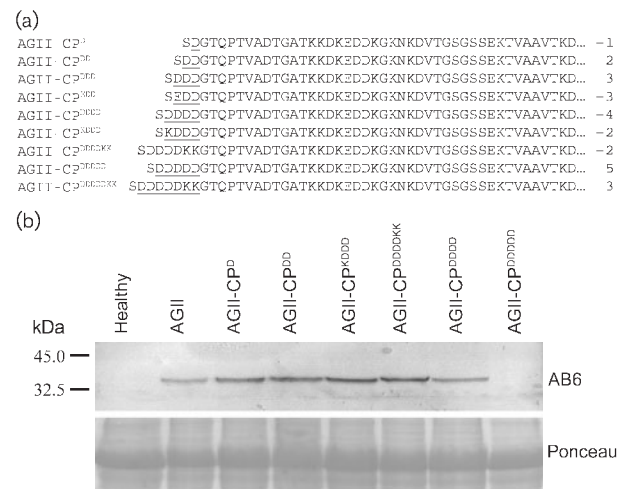


**Fig. 2.** CP accumulation of AGII-CP-Nt positively charged mutants in squash. (a) Schematic representation of the AGII genome organization. AGII non-coding (shaded) and coding (open boxes) regions are shown. The locations of the restriction enzyme sites utilized to construct various AGII CP-Nt mutants are indicated. NIa protease cleavage site is shown by a solidus (/). Foreign residues insertion site in CP is marked by an arrow. (b) CP-Nt amino acid sequence of AGII positively charged mutants. Inserted foreign residues are underlined. CP-Nt predicted net charge for each mutant is indicated on the right. (c) Immunoblot analysis of squash inoculated with AGII positively charged CP-Nt genetically stable mutants 14 days p.i. Total extracts (15  $\mu$ l) of systemic squash leaves were separated by SDS-PAGE (12.5%), blotted and probed with anti-CP AB6 monoclonal antibody. Relative loading of protein in each lane is shown by Ponceau staining. Extracts from non-inoculated (Healthy) plants were used as a negative control. The positions of molecular-mass standards (kDa) are indicated on the left.

(AGII-CP<sup>KKAA</sup>) harbouring two neutral Ala residues instead of Asp upstream of the KKK residues was not infectious (Table 1).

### A CP-Nt net negative charge of up to $-4$ does not abolish virus systemic infectivity

We tested the effect of increased CP-Nt net negative charge on AGII infectivity. This was done by generating AGII mutant cDNA clones engineered to express CP with N-terminal fusion of up to five acidic residues (Fig. 3a). Each mutant cDNA clone was analysed for its ability to support systemic infectivity, its genetic stability and accumulation as described above. Modification of AGII CP-Nt by the addition of one (AGII-CP<sup>D</sup>; Fig. 3a) or two (AGII-CP<sup>DD</sup>; Fig. 3a) Asp residues did not affect virus systemic infectivity (Table 2 and Fig. 3b). N-terminal fusion of three Asp residues to CP-Nt (AGII-CP<sup>DDD</sup>; Fig. 3a) gave a cDNA clone that was rarely infectious



**Fig. 3.** CP accumulation of AGII-CP-Nt negatively charged mutants in squash. (a) CP-Nt amino acid sequence of AGII negatively charged mutants. Inserted foreign residues are underlined. CP-Nt predicted net charge for each mutant is indicated on the right. (b) Immunoblot analysis of squash inoculated with AGII negatively charged CP-Nt genetically stable mutants 14 days p.i. Total extracts (20  $\mu$ l) of systemic squash leaves were separated by SDS-PAGE (12.5%), blotted and probed with anti-CP AB6 monoclonal antibody. Relative loading of protein in each lane is shown by Ponceau staining of a similar membrane. The positions of molecular-mass standards (kDa) are indicated on the left.

(Table 2). This mutant was not genetically stable and only virus progeny harbouring one base pair mutation that convert Asp to Ala were recovered (Table 2). However, an N-terminal fusion of an EDD peptide to CP-Nt (AGII-CP<sup>EDD</sup>; Fig. 3a) gave a partially infectious cDNA clone. In this case, genetically stable and unstable virus progeny were detected (Table 2). The genetically unstable progeny had a single base pair mutation converting the added Glu to Lys (SKDDGTQ). We then engineered the non-stable AGII-CP<sup>DDD</sup> clone to include one positively charged Lys residues upstream of the added Asp residues (AGII-CP<sup>KDDD</sup>; Fig. 3a). This addition is predicted to change the CP-Nt net charge from  $-3$  to  $-2$ , similar to CP-Nt net charge of systemically infectious AGII-CP<sup>DD</sup> (Table 2). Indeed, AGII-CP<sup>KDDD</sup> was systemically infectious 7–8 days p.i., accumulated as parental AGII and was genetically stable (Table 2 and Fig. 3b). An additional mutant AGII-CP cDNA clone, engineered to have a CP-Nt net charge of  $-4$  by fusion of four Asp residues (AGII-CP<sup>DDDD</sup>; Fig. 3a), was infectious and genetically stable but had a 7 day delay in symptom appearance (Table 2) and a decreased CP accumulation (Fig. 3b). Here, again, addition of two basic Lys residues to AGII-CP<sup>DDDD</sup> CP-Nt, changing its net charge from  $-4$  to  $-2$  (AGII-CP<sup>DDDDKK</sup>; Fig. 3a) improved infection kinetics (Table 2) and CP accumulation (Fig. 3b). Fusion of five Asp residues to CP-Nt (AGII-CP<sup>DDDDD</sup>) abolished virus infectivity completely and no

**Table 2.** Characterization of AGII negatively charged CP-Nt mutants

Clone	CP-Nt pI/net charge*	Systemic infectivity (%)†	Time of symptom appearance (days p.i.)	Progeny CP-Nt sequence‡	Progeny CP-Nt pI/net charge*
AGII-CP	6·09/0	100	6–7	SGTQ	6·09/0
AGII-CP <sup>D</sup>	5·17/–1	100	7	SDGTQ	5·17/–1
AGII-CP <sup>DD</sup>	4·85/–2	100	7–8	SDDGTQ	4·85/–2
AGII-CP <sup>DDD</sup>	4·65/–3	11/NO	–	SDADGTQ	4·85/–2
AGII-CP <sup>EDD</sup>	4·69/–3	42	10	SEDDGTQ	4·69/–3
				SKDDGTQ	5·20/–2
AGII-CP <sup>KDDD</sup>	4·88/–2	100	7–8	SKDDDGTQ	4·88/–2
AGII-CP <sup>DDDD</sup>	4·51/–4	100	13–15	SDDDDGTQ	4·51/–4
AGII-CP <sup>DDDDKK</sup>	4·92/–2	100	8–10	SDDDDKKGTTQ	4·92/–2
AGII-CP <sup>DDDDD</sup>	4·41/–5	0	–	–	–
AGII-CP <sup>DDDDDKK</sup>	4·73/–3	33	15	SDDDDDKKGTQ	4·73/–3
				SNDDDDKKGTQ	4·92/–2

\*Predicated isoelectric point (pI) was determined using ExPASy ProtParam tool (<http://au.expasy.org/tools/protparam.html>). Net charge = total number of positively charged residues (Lys and/or Arg) minus total number of negatively charged residues (Glu and/or Asp).

†Systemic infectivity of indicated clone on squash plants (at least nine plants tested). 0, no virus accumulation and visible symptoms were observed. NO, RT-PCR and sequencing analysis could not recover the original clone sequence.

‡As determined by RT-PCR of virus progeny and direct sequencing of amplified product. Bold residues are those differing from originally inoculated constructs.

infectious mutant progeny could be recovered (Table 2 and Fig. 3b). Addition of two Lys residues to AGII-CP<sup>DDDD</sup> CP-Nt (AGII-CP<sup>DDDDKK</sup>; Fig. 3a), changing CP-Nt net charge from –5 to –3, rescued virus infectivity although infectivity was delayed (Table 2). RT-PCR and sequence analysis of progeny viruses from the infected plants occasionally revealed a single nucleotide change that substituted one of the added Asp to Asn (Table 2). This spontaneous substitution changed CP-Nt net charge of infectious viral progeny from –3 to –2.

### Effect of CP-Nt charge mutations on virus assembly and movement

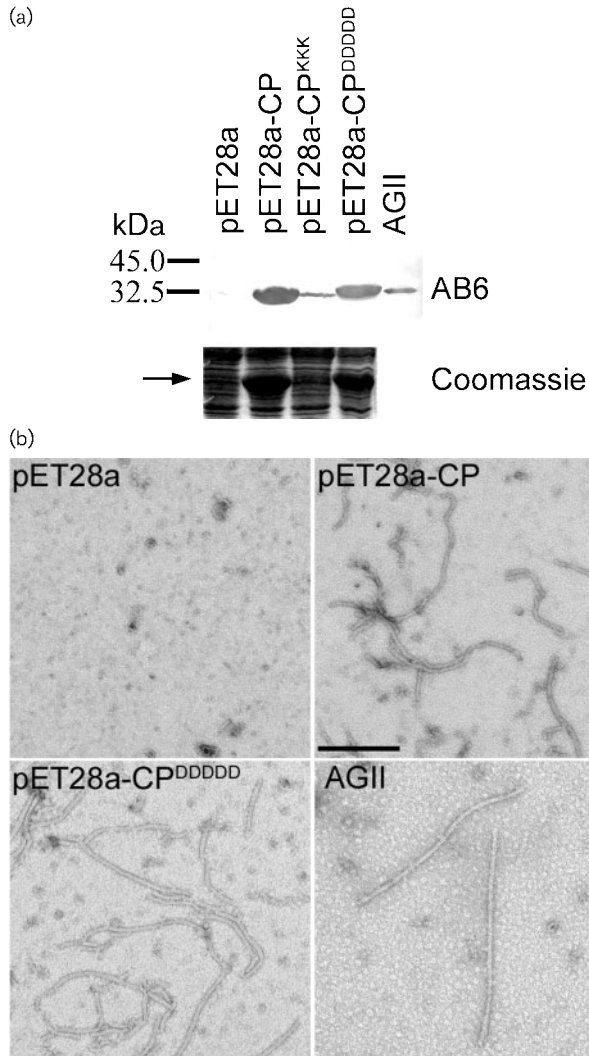
To determine the biological reason for lack of infectivity of AGII-CP<sup>KKK</sup> and AGII-CP<sup>DDDDD</sup>, we tested their ability to assemble into a virion and to spread from cell-to-cell. Assembly was tested by assaying the ability of recombinant CP<sup>KKK</sup> and CP<sup>DDDDD</sup>, expressed in *E. coli* to self assemble into PVLPS. Wild-type CP and CP<sup>DDDDD</sup> were partially expressed as soluble proteins (Fig. 4a), however, CP<sup>KKK</sup> was expressed almost solely as a non-soluble protein and only a very small fraction could be detected as soluble (Fig. 4a). The presence of PVLPS in each bacterial extract was confirmed by visualization under the electron microscope. PVLPS were readily observed in CP and CP<sup>DDDDD</sup> soluble extracts (Fig. 4b) but not in CP<sup>KKK</sup>, probably due to its low concentration in the soluble extract. Efforts to increase CP<sup>KKK</sup> concentration by solubilization from inclusion bodies and ultrafiltration were not successful.

The ability of AGII-CP<sup>KKK</sup> and AGII-CP<sup>DDDDD</sup> to spread from cell-to-cell was monitored by insertion of a GFP tag

between the NIb and CP genes (Fig. 5a). Particle bombardment was used to introduce the GFP-tagged viruses into cotyledon epidermal cells of squash. In the wild-type clone (AGII-GFP), strong GFP fluorescence was detected in the cytosol and nucleus of inoculated cotyledon epidermal cells 4–5 days p.i. (data not shown). At 6 days p.i., GFP fluorescence covered large areas of the bombarded cotyledons suggesting that AGII-GFP was moving from cell-to-cell (Fig. 5). At 10 days p.i., GFP fluorescence continued to spread from cell-to-cell and through the phloem to upper systemic leaves (Fig. 5) confirming that AGII-GFP is fully infectious. AGII-GFP-CP<sup>KKK</sup> and AGII-GFP-CP<sup>DDDDD</sup> charged mutants showed the same defective movement phenotype with GFP restricted to single cells only at 6 days p.i. (Fig. 5). At 10 days p.i., very limited spreading to neighbouring cells was observed in both mutants (Fig. 5).

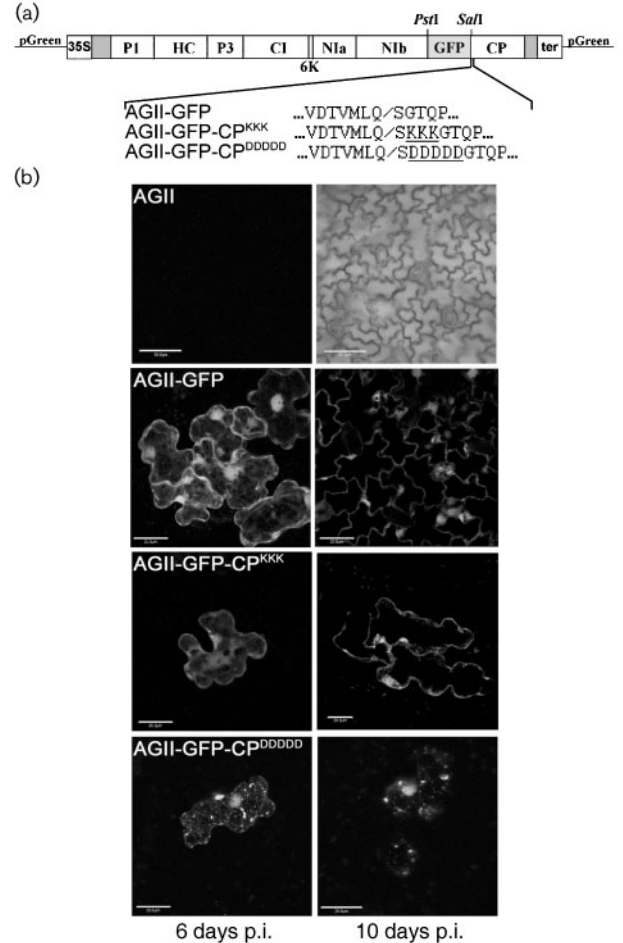
### DISCUSSION

We have shown before that ZYMV CP-Nt is not essential for assembly or movement (Arazi *et al.*, 2001a). Strangely, a deletion of only 20 aa from CP-Nt (AGII-CPΔ20) abolished systemic infectivity raising the possibility that a CP-Nt characteristic, other than sequence, plays a role in virus systemic infectivity. The ZYMV-AGII CP-Nt is 43 aa long and contains an equal number of positively and negatively charged amino acid residues (two Glu, six Asp and eight Lys; Fig. 1a) giving a net charge of 0 and a predicted isoelectric point (pI) of 6·09. Calculation of CP-Nt net charge for CP-Nt deletion mutants characterized previously [AGII-CPΔ8 (0) and AGII-CPΔ13 (+1)] (Arazi



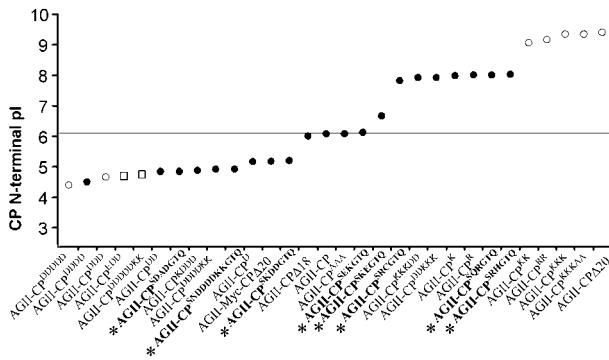
**Fig. 4.** Expression of CP<sup>KKK</sup> and CP<sup>DDDDD</sup> recombinant proteins in bacteria and formation of PVLPs. (a) Immunoblot analysis of soluble extracts of *E. coli* expressing wild-type AGII CP (pET28a-CP), recombinant CP<sup>KKK</sup> (pET28a-CP<sup>KKK</sup>) or recombinant CP<sup>DDDDD</sup> (pET28a-CP<sup>DDDDD</sup>). Extracts (10  $\mu$ l) were analysed on SDS-PAGE (12.5%), blotted and probed with anti-CP AB6 monoclonal antibody. CP from purified AGII particles was used (AGII) as a mobility reference. Relative loading of protein in each lane is shown by Coomassie staining. The positions of molecular-mass standards (kDa) are indicated on the left. (b) Electron micrographs of the PVLPs formed in the above soluble extracts. AGII virus particles are also shown as a reference (AGII). Bar, 370 nm.

*et al.*, 2001a) and in this study [AGII-CP $\Delta$ 18 (0), AGII-CP $\Delta$ 20 (+2) and AGII-CP $\Delta$ 33 (0)] revealed that AGII-CP $\Delta$ 20, the only non-infectious deletion mutant examined, has the highest CP-Nt net charge. Indeed, infectivity was restored upon neutralization of the excess positive charge by fusion of either two negatively charged Asp residues [AGII-CP $\Delta$ 18 (0)] or a foreign Myc peptide [AGII-Myc-CP $\Delta$ 20 (-1)], suggesting that CP-Nt net charge and not



**Fig. 5.** Localization of GFP fluorescence in squash cotyledons. (a) Schematic representation of GFP-tagged AGII and its charged mutants. Inserted charged residues are underlined. Nla protease cleavage site is shown by a solidus (/). AGII non-coding (shaded) and coding (open boxes) regions including the inserted GFP gene are shown. The locations of CaMV 35S promoter (35S) and NOS terminator (ter) and the restriction enzyme sites utilized to insert the GFP gene into AGII coding region are indicated. (b) Representative GFP images of AGII-GFP, AGII-GFP-CP<sup>KKK</sup> and AGII-GFP-CP<sup>DDDDD</sup> inoculated cotyledons 6 and 10 days p.i. are shown (Bar, 20  $\mu$ M). As a negative control, GFP (left) and transmitted light (right) images of a cotyledon bombarded with AGII plasmid DNA are shown (Bar, 50  $\mu$ M).

sequence is an important determinant of virus systemic infectivity. This conclusion is reinforced by our analysis of positively charged mutants, as summarized in Table 1. This analysis demonstrates that systemic infectivity and genetic stability of AGII is disrupted specifically when CP-Nt net charge is raised above +1 ( $p > 8 \cdot 03$ ; AGII-CP<sup>KK</sup>, AGII-CP<sup>RR</sup> and AGII-CP<sup>KKK</sup>). Furthermore, lowering CP-Nt net positive charge of AGII-CP<sup>KKK</sup> to +1 ( $p < \sim 8 \cdot 03$ ) by fusion of acidic Asp residues restores virus systemic infectivity and genetic stability (AGII-CP<sup>KKKDD</sup>



**Fig. 6.** Predicted pI of CP-Nt of mutants described in this study. Mutant progeny formed due to genomic instability are marked by an asterisk (\*). pI values of infectious genetic stable or unstable mutants are marked by a filled circle or an open square, respectively. pI values of non-infectious mutants or mutants that could not be recovered due to genetic instability are marked by an open circle.

and AGII-CP<sup>DDKKK</sup>). Infectivity was also restored by spontaneous mutations of AGII-CP<sup>KK</sup> and AGII-CP<sup>RR</sup> virus progeny *in planta*, which substitute CP-Nt foreign basic residues for Glu or non-charged residues, lowering its net charge to either +1 or 0 (Fig. 6 and Table 1). Similarly, spontaneous mutations that reduced foreign insert pI were previously reported in CPMV (Porta *et al.*, 2003). We have previously observed that fusion of a highly basic *Foot-and-mouth disease virus* (FMDV) CP peptide to a 13 aa truncated AGII CP-Nt (AGII-FMDVΔ13) resulted in a non-infectious clone (Arazi *et al.*, 2001a) and that this clone becomes infectious upon N-terminal fusion of an acidic Myc peptide (AGII-Myc-FMDVΔ13; Arazi *et al.*, 2001a). Calculation of CP-Nt net charge of AGII-FMDVΔ13 (+4; pI=9.70) and AGII-Myc-FMDVΔ13 CP-Nt (+1; pI=7.95) provides an explanation to these observations in accordance with our present data (Fig. 6). Hence, the principles we have drawn in this study are also expected to improve our ability to express charged foreign epitopes on the surface of AGII. This should be done by taking hybrid CP-Nt net charge into consideration and adjusting it by fusing neutralizing residues where needed.

It has been shown that substitutions of basic Lys or Arg for acidic Asp in CP-Nt DAG motif of TVMV and TEV abolished systemic infection of tobacco suggesting that charge changes done specifically in the DAG motif alter virus capacity for systemic movement (Lopez-Moya & Pirone, 1998). We calculated that the CP-Nt net charge of TVMV was modified from 0 (pI=6.01) to +2 (pI=9.30) and of TEV from -1 (pI=5.04) to +3 (pI=9.52) following mutagenesis. These CP-Nt net charges are consistent with the values that did not support AGII systemic infectivity (Fig. 6). The fact that AGII infectivity was abolished and recovered either by fusion of charged residues to a full-length CP-Nt or truncated domain lacking the DAG

motif, suggests that at least in ZYMV, infectivity is affected by global CP-Nt net charge and not by a specific charge change done in a specific location of this domain. Calculation of CP-Nt net charge from 28 different potyvirus CP amino acid sequences listed and aligned in Shukla *et al.* (1994) shows that none possess a net charge larger than +1, although aligned CP-Nts are highly variable in sequence and length (21–96 aa) (Supplementary Table A). This finding may indicate that the limit of CP-Nt net positive charge that we have defined for ZYMV infectivity might hold for other potyviruses as well.

Our data suggest that ZYMV is less affected by negatively charged residues added to CP-Nt. We have demonstrated that up to four negatively charged residues can be fused to CP-Nt without abolishing systemic infection (AGII-CP<sup>DDDD</sup>). Nevertheless, this fusion resulted in a delayed systemic infection (Table 2) and reduced virus accumulation (Fig. 3b), indicating that it was not optimal for virus life-cycle. In addition, AGII mutants with CP-Nt net charge of -3 (AGII-CP<sup>DDD</sup>, AGII-CP<sup>EDD</sup> and AGII-CP<sup>DDDDDKK</sup>) were, in general, genetically unstable (Table 2). This result is enigmatic because of the infectivity and genetic stability of AGII-CP<sup>DDDD</sup> (-4) and AGII-Myc-CPΔ33 (-3) clones. One possible explanation is that in these clones some of CP-Nt negative charge was masked, resulting in an actual higher CP-Nt net charge, which allowed systemic infection. Indeed, addition of less than three acidic residues to CP-Nt (net charge > -3) does not affect virus systemic infectivity. This is also supported by progeny viruses of unstable clones, which mutated CP-Nt to reduce its net charge to either -1 or -2 (Table 2). Moreover, fusion of basic Lys residues, which partially neutralizes CP-Nt net charge [AGII-CP<sup>KDDD</sup> (-2), AGII-CP<sup>DDDDKK</sup> (-2) and AGII-CP<sup>DDDDDKK</sup> (-3)], considerably improved virus infectivity and accumulation.

Currently, we cannot determine the exact reason for lack of infectivity upon change of CP-Nt net charge. Formation of ZYMV PVLPS in bacteria following expression of recombinant CP<sup>DDDDD</sup> protein suggest that the change of CP-Nt net charge to negative does not affect particle assembly. However, we cannot exclude the possibility that a net positive charge of CP-Nt disturbs virus assembly *in planta*. Nevertheless, in TEV and TVMV, substitutions that involved positively charged amino acids and increased CP-Nt net charge above +1 affected virus movement and not assembly or replication (Lopez-Moya & Pirone, 1998). Indeed, AGII-GFP loses its ability to move efficiently from cell-to-cell upon fusion of charged residues to its CP-Nt (AGII-GFP-CP<sup>KKK</sup> and AGII-GFP-CP<sup>DDDDD</sup>; Fig. 5), suggesting that a neutral CP-Nt net charge may be required to enable efficient potyviral cell-to-cell movement. Interestingly, the deleterious effect of high CP pI on virus infectivity was already demonstrated in TMV and CPMV where expression of very basic immunogenic peptides (pI ~ 10.81 and ~ 12.00) as fusions with their CPs reduced chimaeric virus infectivity by promoting cell death in TMV

(Bendahmane *et al.*, 1999) and restricting long distance movement in CPMV (Porta *et al.*, 2003). Like in ZYMV, in TMV and CPMV basic peptides were expressed in CP regions known to be highly exposed on the virus surface. Thus, an exposed positive charge abolished systemic infectivity in three distinct virus families. This charge might disturb an interaction with an unknown host component, which is essential for virus cell-to-cell or systemic movement.

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