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# Multiple pathogenic factors influence aphid transmission of cauliflower mosaic virus from infected plants

Nadia S. Al-Kaff, David S. Turner and Simon N. Covey*
*Department of Virus Research, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK (Fax: +44 1 603 456844); *Author for correspondence*

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**Abstract**

Aphid transmission of cauliflower mosaic virus (CaMV) is mediated by a polypeptide (P18) encoded by the viral gene II. We have investigated the factors which influence acquisition by aphids of CaMV variants from infected plants. Aphid non-transmissible ($AT^-$) CaMV isolates with a full-length gene II sequence share two amino acid changes, *gly* to *arg* at position 94 and *ile* to *val* at 105, relative to wild type transmissible ($AT^+$) isolates. We have mutated the *gly* to *arg* at position 94 in the $AT^+$ isolate Cabb B-JI which then exhibited the $AT^-$ phenotype as predicted. However, replacement of a DNA fragment in Cabb B-JI with one containing the *gly* to *arg* change from the $AT^-$ isolate Campbell to produce hybrid pBJIC1 resulted in a change in symptom phenotype as well as in aphid transmissibility. pBJIC1 also showed characteristics of partial transmissibility related to the stage of infection when it was tested. The level of P18 was measured in plants and showed that recombinants based upon the Campbell ($AT^-$) genome accumulated P18 later than those based upon the Cabb B-JI genome ($AT^+$). However, the Campbell P18 or recombinant proteins like it, were still not able to mediate transmission even when the P18 level in plants was relatively high and by employing large numbers of aphids. We conclude that aphid transmissibility of CaMV is influenced by multiple factors including P18 levels, inherent functionality of the protein, pathogenic characters of the infecting strain, and the number of aphids used to test transmissibility.

*Abbreviations:* $AT^{+/-}$ – aphid-transmissible/non-transmissible.

## Introduction

Cauliflower mosaic virus (CaMV) is a plant DNA virus that is naturally transmitted from plant to plant by aphids in a semi-persistent manner (Markham et al., 1987). There has been considerable interest in the biological and molecular factors controlling acquisition and transmission of CaMV by aphids since the realization that this function is specified by the viral genome (Lung and Pirone, 1974; Woolston et al., 1983; Armour et al., 1983). Of six identified CaMV genes (see Figure 1A), gene II is known to encode an 18000 mol wt polypeptide (P18) that is required for acquisition of CaMV by aphids (Woolston et al., 1983; Armour et al., 1983). P18 is found in infected plants predominantly in electron-lucent sub-cellular inclusion bodies which also contain some virus particles (Espinoza et al., 1991).

Aphid transmissible CaMV strains are designated genetically as $AT.^+$ However, a number of variants have been isolated from natural populations of CaMV that are defective in aphid transmission and designated $AT.^-$ Two $AT^-$ isolates have deletions in gene II of 421 bp in CM4–184 (Howarth et al., 1981) and 332 bp in 11/3–3 (Al-Kaff and Covey, 1994). Three further $AT^-$ CaMV isolates have been identified, CM1841, Campbell and 11/3–7, that have full-length gene II sequences (Hull, 1980; Woolston et al., 1983; Al-Kaff and Covey, 1994). Whilst the reason for lack of transmissibility of the CaMV variants with deletions in gene II is apparent, that for isolates with a full-length gene is less obvious. By constructing recombinants between $AT^+$ and $AT^-$

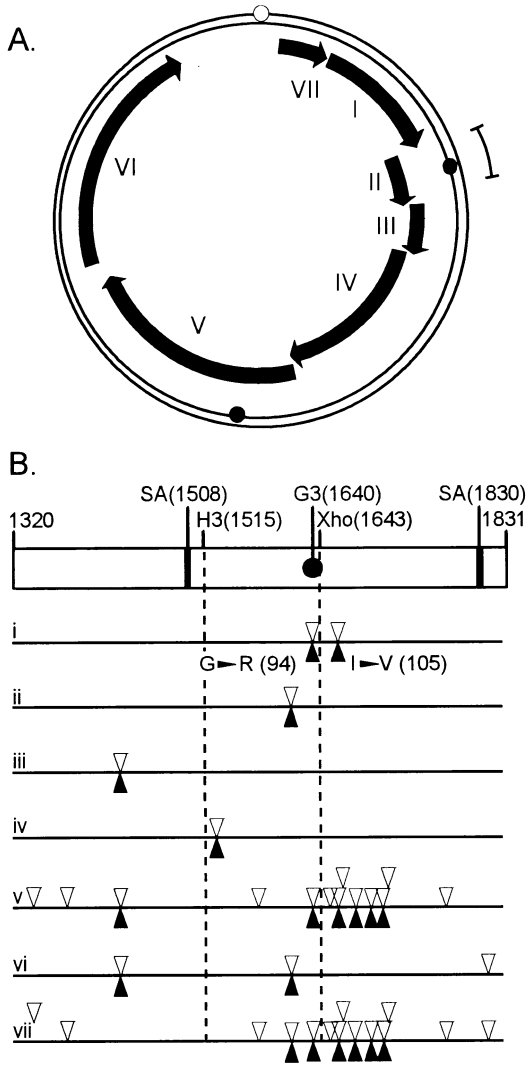


*Figure 1*. A. Map of the CaMV genome showing gene II encoding the aphid transition polypeptide P18 in relation to the other viral genes (black arrows). Replication priming sequences include the (–) strand primer (open circle) and the two (+) strand primers (closed circles) one of which is located in gene II. B. Expanded view of the gene II open reading frame and associated features (denoted by nucleotide positions) including the plus-strand primer (G3), putative splice acceptor (SA) sequences and restriction enzyme sites (H3, *Hin*dIII; Xho, *Xho*I). The lines below summarize amino acid (closed triangle) and nucleotide (open triangle) changes that are common only to (i) AT$^-$ CaMV variants, (ii) unique to CM1841, (iii) unique to Campbell, (iv) unique to 11/3–7, (v) differences between Campbell and Cabb B-JI, (vi) differences between Campbell and CM1841, (vii) differences between CM1841 and Cabb B-JI.

isolates, and from comparisons of gene II nucleotide sequences, Woolston et al. (1987) concluded that a *gly* to *arg* substitution at amino acid position 94 was responsible for the lack of transmissibility in these variants. However, the gene II protein from the AT$^-$ isolate CM1841 was found to have a functional protein *in vitro* (Blanc et al., 1993). Analysis of the CM1841 protein in infected plants indicated that it was relatively unstable and declined after the initial stages of infection suggesting that the AT$^-$ phenotype was due to low levels of an otherwise transmission-capable polypeptide (Nakayashiki et al., 1993). This implies that other AT$^-$ isolates with a full-length gene II sequence might behave in a similar manner to CM1841.

Woolston et al. (1987) also showed that aphid transmissibility of the AT$^-$ isolate Campbell could be restored by replacing a 129 bp fragment containing the *gly* to *arg* mutation with the equivalent fragment from the AT$^+$ CaMV isolate Cabb B-JI. Although the level of P18 was not measured in the AT$^+$ recombinant, it is suggested that the exchanged fragment might have improved the stability of the P18 in infected plants rather than altering its transmission properties *per se*. We have now investigated the aphid transmission properties of the Campbell isolate further and have shown that although a single amino acid change in gene II is apparently sufficient to abolish transmissibility, we have found that multiple factors are involved in determining whether or not CaMV is transmitted by aphids.

## Materials and methods

### *Virus and plants*

CaMV isolates used in this study were Cabb B-JI, Aust, Bari–1 and Campbell (Al-Kaff and Covey, 1994, 1995). The plant used were *Brassica rapa-rapifera* (turnip) cv. 'Just Right' and *Brassica oleracea-gongylodes* (kohlrabi) cv. 'Purple Vienna'. Plants were mechanically inoculated at the two leaf stage by rubbing infectious sap, or DNA onto the second leaf with celite abrasive. Plants were propagated in a containment glasshouse at 18–22 °C in a minimum 16 h photoperiod.

### *Aphid transmission*

Aphid transmissibility of CaMV was tested using the aphid *Myzus peresicae*. Aphids were starved for 2–2.5 h and then fed for 2–2.5 h on young leaves freshly-harvested from infected plants, and placed in a Petri dish on dampened filter paper. Aphids were then counted and transferred to healthy plants 10–15 days old which were then covered to prevent aphid movement

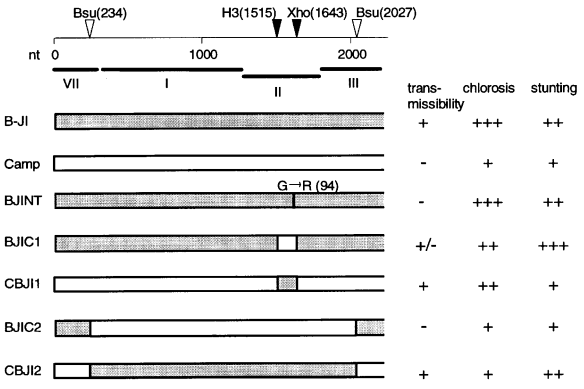


*Figure 2*. CaMV mutant and recombinants between CaMV isolates Cabb B-JI (filled areas) and Campbell (open areas). Biological features of the infections are shown with increased severity expressed as more crosses.

to other plants. Aphids were left to feed for 24 h and then killed by fumigation. Plants were transferred to a glasshouse as above.

*Construction of hybrid virus genomes and mutagenesis*

Starting clones were pCa33 (Woolston et al., 1987) and pCa24 which contained infectious genomes of isolates Campbell and Cabb B-JI, respectively. Hybrid pHy14 (Woolston et al., 1987), kindly provided by Dr C. Woolston, was re-cloned into bacterial vector pGEM7 and renamed pCBJI1. Clone pBJIC1, a reciprocal hybrid to pCBJI1, in which the *Hin*dIII(1515)-*Xho*I(1643) fragment from Campbell replaced that in the Cabb B-JI genome, was constructed by exchanging the fragment in a *Spe*I(109)-*Xho*I(1643) subclone. Clones pBJIC2 (mainly Cabb B-JI) and pCBJI2 (mainly Campbell) were reciprocal hybrids constructed by exchanging *Bsu*36I fragments (nucleotides 234–2027) between the respective parental isolates.

Mutagenesis of the Cabb B-JI genome in gene II at amino acid position 94 to substitute an *arg* for a *gly* residue was performed on a *Spe*I(109)-*Xho*I(1643) subclone by changing a G nucleotide to an A at position 1629 using the PCR mutagenesis method of Ho et al. (1989) followed by replacement of a *Hin*dIII(1515)-*Xho*I(1643) containing the mutated sequence. The structures of the above genomes are shown in Figure 2.

*Detection of P18*

Inclusion bodies were isolated from infected turnip plants as described by Woolston et al. (1983) and P18 was detected by immuno-blotting following polyacrylamide gel electrophoresis using an antiserum raised against purified P18 kindly provided by Dr R. Hull.

## Results

*A single base change in gene II causes loss of aphid transmissibility*

Organisation of the CaMV genome and that of gene II, specifying aphid transmissibility, are shown in Figure 1. Sequence comparison of 21 CaMV isolates and clones (Al-Kaff and Covey, 1994) shows 2 nucleotide changes, both causing amino acid substitutions, at positions 94 and 105, respectively, common to the three known CaMV $AT^-$ variants (CM1841, Campbell and 11/3–7) with full-length gene II sequences (Figure 1Bi). Other changes in sequence not found in all $AT^-$ variants are also shown (Figure 1Bii-vii). Woolston et al. (1987) restored aphid transmissibility to the $AT^-$ isolate Campbell by substituting a *Hind*III (1515)-*Xho*I (1643) fragment from the $AT^+$ isolate Cabb B-JI. They predicted that the amino acid change of *gly* to *arg* at position 94 in the gene II protein was responsible for the $AT^-$ phenotype in Campbell. To test this, we used site-directed mutagenesis to alter the nucleotide G to an A at 1629 in Cabb B-JI (to give clone pBJINT, see Figure 2) causing the change in the protein. Plants inoculated with pBJINT DNA developed smaller local lesions but systemic symptoms were similar to those of wild type Cabb B-JI infections. Aphid transmissibility was tested by feeding 50 aphids on each of 10 plants infected by pBJINT. None of the plants subsequently probed by the aphids developed symptoms. This demonstrated that the *gly* to *arg* substitution at position 94 in the gene II protein had caused loss of transmissibility as expected. Thus, pBJINT had transmission properties similar to those of Campbell and different from Cabb B-JI which showed 100% transmissibility under similar test conditions (Table 1).

Since the *gly* to *arg* change is common to all $AT^-$ isolates with a full-length gene II, we presumed that the reason for lack of transmissibility in Campbell was the same as for CM1841. However, although the CM1841 gene II protein was transmissible *in vitro*,

*Table 1*. Aphid transmissibility of CaMV variants and hybrids

| | A | B | C | D | F | G | H |
|---|---|---|---|---|---|---|---|
| aphids | 10 | 15 | 20 | 50 | 15 | 50 | 200 |
| dpi | 15 | 15 | 15 | 15 | 25 | 25 | 25+ |
| Cabb B-JI | 10/10 | – | 5/5 | 30/30 | – | – | 0/10 |
| Campbell | 0/10 | – | – 0/30 | – | – | – | |
| BJINT | – | – | – | 0/10 | – | – | – |
| BJIC1* | – | 2/10 | – | 2**/70 | 0/10 | 0/10 | – |
| CBJI1 | – | – | – | 30/30 | – | – | – |
| BJIC2 | – | – | – | 0/30 | – | – | – |
| CBJI2 | – | – | – | 30/30 | – | – | – |

Data indicate number of plants infected/number probed by aphids.
* CaMV hybrid showing partial transmissibility.
** Aphid-induced lesions but not systemic symptoms observed.

transmission had not been demonstrated *in vivo* (Blanc et al., 1993). We were interested in the relationship between transmissibility and CaMV pathology particularly since substitution of a single base in gene II of isolate Cabb B-JI had altered both transmissibility and local lesion size. Systemic symptoms produced by pHy14, a recombinant constructed by Woolston et al. (1987) comprising the Campbell genome with a substitution of the *Hind*III (1515)-*Xho*I (1643) fragment from Cabb B-JI, were also different from either parent (C. Woolston. personal communication). We checked this after recloning pHy14 into a different vector plasmid to produce clone pCBJI1 and confirmed that insertion of the Cabb B-JI fragment restored the $AT^+$ phenotype to Campbell. We also found that the systemic leaf chlorosis in plants infected with pCBJI1 was more severe than that of the Campbell parent (Figure 2).

A further hybrid virus was constructed incorporating the *Hind*III (1515)-*Xho*I (1643) fragment from Campbell into Cabb B-JI (creating clone pBJIC1) to compare its properties with the reciprocal recombinant pCBJI1. Plants infected with pBJIC1 were significantly more stunted than either of the parental isolates (Figure 2). Since the substituted fragment contained the same *gly* to *arg* mutation responsible for loss of transmissibility in Campbell, we expected pBJIC1 to be non-transmissible. Unexpectedly, in the first experiment, aphids were able to transmit virus from 2 out of 10 plants infected with pBJIC1. However, when plants infected by the first transmission were subsequently probed with aphids, they were not able to transmit virus to a third set of plants (Table 1). Sequence analysis of viral DNA from the two initially infected plants showed that they had retained the Campbell fragment. This, together with the loss of transmissibility of pBJIC1 following passage by aphids, indicated that the original transmission of pBJIC1 was not due to contamination by a transmissible form of CaMV. This suggested that the hybrid gene II protein exhibited transient transmissibility. Transmission had been achieved initially by probing plants at 15 days post inoculation (dpi) whilst the second probing which failed to transmit virus had been done at 25 dpi. Also, more aphids were used in the second probing to increase the probability of transmission (Table 1). We repeated the experiment seven times, on each occasion using 50 aphids probing plants infected with pBJIC1 at 15 dpi, determining transmission to a total of 70 plants. On only two occasions did we observe transmission to the recipient plants causing asymmetric local lesions adjacent to veins typical of those produced by aphids. However, development of systemic symptoms was not observed in either case.

The transient transmission properties of pBJIC1 suggested that not all of the Campbell $AT^-$ phenotype had been transferred to Cabb B-JI. Moreover, since the single amino acid change at position 94 in pBJINT seemed to confer the $AT^-$ phenotype, this indicated that multiple factors contributed to the $AT^-$ phenotype in plants. Sequences outside of the *Hin*dIII-*Xho*I fragment, possibly including the second amino acid change (*ile* to *val*) common to all $AT^-$ CaMV isolates with a full-length gene II, at position 105, could also be involved. To check this, we substituted a *Bsu36*I fragment (234–2027) containing the complete gene II sequence, from Campbell into Cabb B-JI to give pBJIC2, and constructed its reciprocal pCBJI2 (Figure 2). In three separate experiments, aphids failed to transmit pBJIC2 to a total of 30 plants using 50 aphids per plant at 15 dpi, whereas 100% transmission was observed with pCBJI2 (Table 1). This suggests that elements apart from the *gly* to *arg* change at position 94 might also be involved in the $AT^-$ phenotype. Symptoms produced by pBJIC2 and pCBJI2 also differed from those of their parental isolates, Cabb B-JI and Campbell, respectively (Figure 2) and these different pathogenic characteristics could have had an additional, indirect influence on aphid transmissibility.

*Accumulation of P18 in infected plants*

The explanation for the lack of transmissibility of the $AT^-$ isolate CM1841 from plants was that P18, which was transmissible *in vitro*, appeared to be unstable in plants (Nakayashiki et al., 1993). To determine

whether the transmission properties of our CaMV variants were related to P18 levels, we isolated virus inclusion bodies at three times post inoculation and detected P18 by immuno-blotting following polyacrylamide gel electrophoresis (Figure 3). In plants infected with the transmissible isolate Cabb B-JI, P18 was not detectable in systemically-infected leaves until 14 dpi with the level in plants lower at 21 dpi than at 14 dpi. In contrast, P18 from the non-transmissible isolate Campbell was not detectable until 21 dpi although the level at this time was not significantly lower than that of Cabb B-JI at the same time. This differs significantly from the $AT^-$ isolate CM1841 in which P18 was detected early in infection and then declined (Nakayashiki et al., 1993). The two $AT^+$ recombinants pCBJI1 and pCBJI2 with genomes derived predominantly from Campbell showed P18 accumulation patterns that were similar to those of Campbell in that the protein was first observed relatively late. Similarly, the pattern of accumulation of the $AT^-$ hybrid pBJIC2 was more like that of Cabb B-JI from which the major portion of its genome was derived. The hybrid with both $AT^+$ and $AT^-$ properties, pBJIC1, was unusual in that its P18 accumulated earlier and to higher levels than either of the parental genomes from which it was derived.

*Titration of aphids against transmissibility*

The lack of transmissibility of CaMV isolates with full-length gene II sequences seems to be related to a variety of factors including P18 levels in infected plants, as with CM1841 (Nakayashiki et al., 1993), and other factors affecting the efficiency of aphid acquisition as shown for pBJIC1 (see Figures 2 and 3). The likelihood of successful transmission of a CaMV variant by aphids in an experiment will also depend upon number of aphids employed especially when the virus has relatively poor transmission properties. Since the assignment of a particular virus isolate or mutant as transmissible or not can be critically determined by this combination of factors, we sought to eliminate the aphid as a variable by titration with different numbers of aphids. We compared transmissibility of CaMV $AT^+$ isolates with mild (Bari–1), typical (Cabb B-JI), and severe (Aust) pathogenic characteristics in two host plant species showing susceptible (turnip) and tolerant (kohlrabi) pathogenic responses to infection. The $AT^-$ isolate Campbell was tested only in turnip (Table 2). Aphids were allowed to feed on the test plants at 25–30 dpi and their transmissibility to turnip plants was recorded. With 5 or more aphids per plant, Cabb B-JI was 100% transmissible from turnip but only about 50% transmissible when 1 aphid per plant was employed. However, Cabb B-JI was apparently non-transmissible from kohlrabi plants in which it attains a lower titre than in turnip (Al-Kaff and Covey, 1995) with up to 5 aphids per plant and only 20% transmissible with up to 50 aphids per plant (Table 2). With the mild isolate Bari–1, transmission in turnip was observed with 25 or more aphids per plant and did not reach 100% even with 100 aphids per plant. Bari–1 was barely transmissible from kohlrabi even with 100 aphids per plant. Although CaMV isolate Aust was more severe than Cabb B-JI, it was apparently less transmissible from turnip below 25 aphids per plant but showed a higher level of transmissibility from kohlrabi. The $AT^-$ isolate Campbell was not transmissible from turnip with up to 200 aphids per plant (Table 2).

*Table 2.* Aphid transmissibility of CaMV isolates from turnip (T) and kohlrabi (KR)

| Aphids | Cabb B-JI | | Bari-1 | | Aust | | Camp. |
|---|---|---|---|---|---|---|---|
| | T | KR | T | KR | T | KR | T |
| 1 | 14/30 | 0/30 | 0/30 | 0/30 | 9/30 | 0/30 | – |
| 5 | 10/10 | 0/10 | 0/10 | 0/10 | 6/10 | 0/10 | – |
| 25 | 10/10 | 2/10 | 4/10 | 0/10 | 10/10 | 3/10 | – |
| 50 | 10/10 | 2/10 | 6/10 | 0/10 | 10/10 | 7/10 | 0/10 |
| 100 | – | – | 9/10 | 1/10 | – | – | – |
| 200 | – | – | – | – | – | – | 0/10 |

Data indicate number of plants infected/number probed by aphids.

## Discussion

We have shown that multiple molecular and pathogenic factors combine to determine whether or not an aphid will acquire and transmit CaMV from one host plant to another. The lack of aphid transmissibility from plants of CaMV isolate CM1841, which has a full-length gene II sequence, was suggested to result from relative instability in plants of a P18 transmission factor (Nakayashiki et al., 1993) that was otherwise functional in that it exhibited transmissibility *in vitro* (Blanc et al., 1993). Since Campbell also has a full-length gene II and is very similar to that of CM1841, it would be reasonable to assume that Campbell lacks transmissibility for the same reason as CM1841. Moreover, since the *gly* to *arg* substitution at amino acid 94 is common to all $AT^-$ variants with full-length gene II sequences, and the AT phenotype can be transferred with DNA

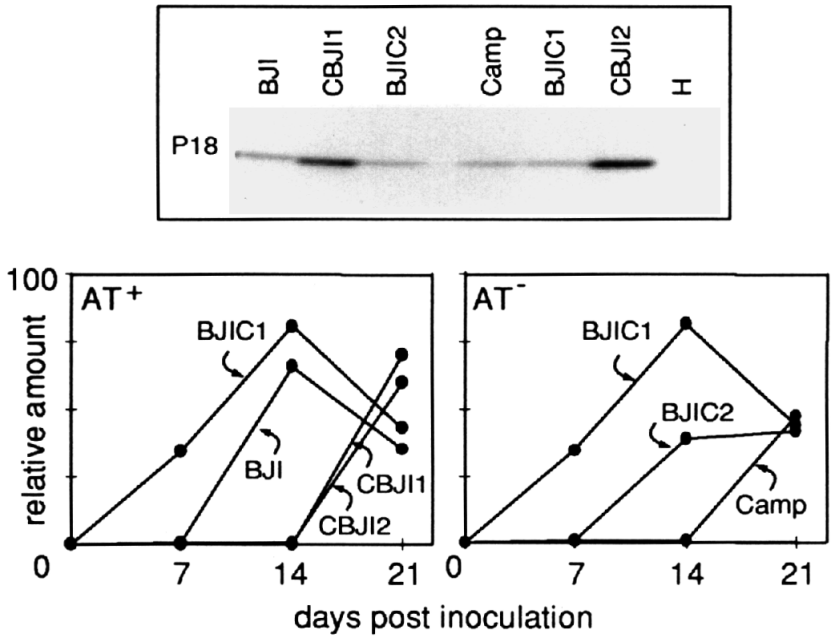


*Figure 3.* Levels of P18 in infected plants. The upper panel shows a typical immuno-blot (taken at 21 days post inoculation) probing for P18 in inclusion body preparations from plants infected with CaMV recombinants (see Figure 2) and a mock inclusion preparation from non-infected plants (H). The lower panels show levels of P18 determined at different times post inoculation. The values were obtained by densitometric scanning of immuno-blots and integration of peaks by computer. The values for the recombinant pBJIC1 are shown in both panels because it has properties of a transmissible and non-transmissible virus.

containing this change, this supports the proposition that the two AT$^-$ isolates lack the function for similar reasons. However, the behaviour of Campbell P18 apparently differs significantly from that of CM1841 in plants in that the former does not show the instability of the latter. This suggests that the instability of CM1841 P18 is probably not an inherent property of the polypeptide but is due to some other viral determinant that lies outside of gene II itself. Alternatively, the three nucleotide differences between CM1841 and Campbell, two causing amino acid substitutions (Figure 1B), could contribute to the different properties of the two proteins.

Our results also strongly suggest that the Campbell P18 has lower affinity for aphids than that of the transmissible CaMV isolate Cabb B-JI when acquiring from plants. We demonstrated the importance of employing a sufficient number of aphids to determine whether or not a particular virus variant was transmissible. However, in none of our experiments were we able to achieve transmission of Campbell from plants even when using up to 200 aphids per plant at a time in infection when the Campbell P18 was relatively abundant. This suggests that transmissibility from plants is influenced by factors not operating during *in vitro* transmission experiments or that the Campbell and CM1841 proteins have different properties themselves. This could be resolved by checking the Campbell P18 transmissibility *in vitro* and studying CM1841/Campbell recombinants *in vivo*.

Notwithstanding the differences discussed above, the *gly* to *arg* substitution common to all AT$^-$ CaMV variants is an important component of the AT$^-$ phenotype. We were able to convert the AT$^+$ isolate Cabb B-JI to an AT$^-$ phenotype by introducing this change to produce mutant pBJINT. However, replacement of the Cabb B-JI *Hin*dIII-*Xho*I fragment with that from Campbell generated a hybrid (pBJIC1) with significantly different properties from either parent or from pBJINT. pBJIC1 produced more severe symptoms in plants (Figure 2) correlated with an elevated level of P18 (Figure 3) and apparently transient aphid transmission properties. We observed a very low level of transmission from plants at a time when the level of pBJIC1 P18 was high. The only other difference between Cabb B-JI and Campbell in the exchanged fragment (apart of the *gly* to *arg* change) is a G to A nucleotide change at 1583 that is amino acid-silent (Figure 1B). If this

difference is important, it must act at the *cis* level which remains a possibility since the recent discovery of splice-acceptor sequences in the gene II region (Kiss-Laszlo et al., 1995).

## Acknowledgements

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