

## Context of the coat protein DAG motif affects potyvirus transmissibility by aphids

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Previous work with tobacco vein mottling virus (TVMV) has established that a highly conserved three amino acid motif, asp-ala-gly (DAG), located near the N terminus of the coat protein (CP), is important for aphid transmission. However, several other potyviruses which have motifs other than DAG are aphid-transmissible. Creation of these motifs in TVMV through site-directed mutagenesis failed to render TVMV aphid-transmissible from infected plants, and the creation of a putative complementary motif in the helper component did not restore transmissibility. In an isolate of tobacco etch virus (TEV) that contains two consecutive DAG motifs separated by a single ala, transmissibility was abolished or reduced by mutations affecting the first motif, whereas mutations in the second motif had little or no effect. In a TEV mutant made non-transmissible due to an altered first motif, substitution of val for ala in the position immediately before the second DAG restored transmissibility, whereas changing val to ala in the location prior to the first DAG resulted in reduced TEV transmissibility. In contrast, a val to ala change in the position preceding the single DAG motif of TVMV did not affect transmission. Creation of another DAG motif at the beginning of the TVMV CP core, in a position where certain other potyviruses have a second DAG motif, did not restore transmissibility. Our results suggest that the mere presence of a DAG motif does not guarantee transmissibility and that the context in which the DAG or equivalent motif is found plays a role in the process.

### Introduction

Potyvirus transmission by aphids involves at least two virus-encoded proteins, the coat protein (CP) and the helper component (HC) (Pirone, 1991). Harrison & Robinson (1988) proposed that a conserved motif, asp-ala-gly (DAG), located near the N terminus of the CP of many potyviruses, plays a role in the transmission process. This hypothesis was supported by site-directed mutagenesis analysis in tobacco vein mottling virus (TVMV), which showed that most substitutions and all deletions within the motif resulted in loss or drastic reduction of transmissibility (Atreya *et al.*, 1990, 1991, 1995).

In spite of the characteristic variability of the CP N-terminal region (Shukla & Ward, 1989), comparison of potyvirus CP sequences showed that the DAG motif is highly conserved in aphid-transmissible isolates and that loss of transmissibility is

often correlated with changes in the DAG motif (Atreya *et al.*, 1991). However, there are several reports of aphid-transmissible potyviruses that contain motifs other than DAG. An aphid-transmissible isolate of pea seed-borne mosaic virus (PSbMV) contains a DAS motif (Johansen *et al.*, 1996). Peanut mottle virus (PeMoV) has a DAA motif (Flasinski & Cassidy, 1998), which in TVMV was associated with nearly complete loss of transmissibility (Atreya *et al.*, 1995). In plum pox virus (PPV) isolate 3.3, a 15 amino acid deletion that included deletion of the G in the DAG motif, resulting in a DAL motif, was transmitted when supplemented with active potato virus Y HC (López-Moya *et al.*, 1995). In this study we reproduced these motifs in TVMV through site-directed mutagenesis and studied the effect of the presence of DAS, DAA or DAL motifs on aphid transmissibility from infected plants and also in membrane-acquisition experiments with purified virions supplemented with purified TVMV HC.

Although the amino acid in the position immediately following the DAG motif is not highly conserved among potyviruses, substitution of certain amino acids in this position adversely affected transmission of TVMV (Atreya *et al.*, 1991,

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1995). This suggested that the context within which the DAG lies can affect transmission. To further study the effect of the context we used a highly aphid-transmissible (HAT) isolate of tobacco etch virus (TEV). The N-terminal sequence of TEV is similar to that of TVMV, and the HAT isolate has an additional DAG separated from the first by an ala residue. Our initial goals were to establish which of these two consecutive motifs was required for transmissibility and to compare the results with the previously established transmissibility requirements in the CP of TVMV. To complete our observations, several additional mutations were produced to substitute the amino acid immediately prior to each motif in TEV or prior to the unique motif in TVMV.

Sequence comparison revealed a second DAG motif at the beginning of the CP core (Shukla & Ward, 1989) in some potyviruses, including PSbMV and PPV. A DAG motif was created at this position in the TVMV CP to determine whether this would affect the transmissibility of TVMV variants in which the sequences DAG, DAL or DAS were present in the first motif.

In a recently published study, Flasiński & Cassidy (1998) suggested that a compatible HC sequence might allow transmission of viruses with CP motifs other than DAG. These authors compared the transmission efficiency obtained with several potyvirus CP/HC combinations, including PeMoV, and found that maximum transmission efficiency was obtained with homologous combinations. Their comparison of the HCs of 16 distinct potyvirus species revealed the presence of a conserved CCC sequence; in PeMoV the corresponding sequence is ASC. They suggested that the homologous combination of DAA in the CP and ASC in the HC might be responsible for efficient transmission of PeMoV. We found that the sequence of PSbMV HC (CP motif DAS) in this region is CSC, compatible with their hypothesis. Accordingly, we created a CSC motif in the HC of the TVMV DAS mutant to determine its effect on transmissibility.

## Methods

■ **Full-length clones and sequences.** Genome-length cDNA clones of two potyviruses, TVMV and TEV, were used. Plasmid pXBS7 contains a cDNA copy of the TVMV genome with a T7 promoter and an *Sst*I restriction site located after the poly(A) tail for linearization (Domier *et al.*, 1989). Plasmid pTEV-7DA, a modified version of pTEV-7D (Dolja *et al.*, 1992), was kindly provided by J. C. Carrington (Washington State University, USA). This plasmid is a TEV genomic cDNA clone with an SP6 promoter and a *Bgl*II site for linearization downstream of its poly(A) tail. All numbering of the TVMV and TEV genomes follows Domier *et al.* (1986) and Allison *et al.* (1986), respectively.

■ **In vitro mutagenesis.** TVMV CP mutants were obtained by site-directed mutagenesis (Kunkel *et al.*, 1987) in a *Sall*-*Spe*I subclone (nucleotide positions 7883 to 9251) of pXBS7. After identification of clones containing the mutations, the fragments were replaced in the corresponding sites of pXBS7 as described previously (Atreya *et al.*,

1995). The *Sall*-*Spe*I subclone was also used to introduce two mutations (asp for asn and ala for thr at positions 33 and 34 in the CP), which created a new DAG motif at the beginning of the CP core, where some other potyviruses have DAG motifs. To engineer these changes PCR was used to create a new *Kpn*I restriction site at nucleotide position 8528 without changing amino acids. A *Mun*I to *Spe*I (position 8493 to 9251) fragment was then used to introduce the second DAG motif in each one of the corresponding subclones to create the DAG-DAG, DAL-DAG and DAS-DAG constructs.

The alteration of the motif CCC to CSC in TVMV HC was made by a T to A change at nucleotide 1847 in an *Acc*I subclone of pXBS7. The CSC-containing *Acc*I subclone was ligated to pES6 (Atreya *et al.*, 1992) and the *Acc*I-pES6 clone was then cut at an *Mlu*I site at nucleotide 3872 and ligated to the *Mlu*-*Sst*I fragment of the plasmid containing the DAS CP mutation. This created a full-length construct that contained both the CSC HC and the DAS CP motifs.

Sequence analysis of the CP coding region of pTEV-7DA revealed two nucleotide substitutions (G for A and T for C at positions 8530 and 8539, respectively), resulting in two amino acid changes (gly for asp and val for ala at positions 5 and 8 in the CP) when compared with the published sequence. The resulting N-terminal sequence of the CP is SGTVGAGVDAGKKK ... The N-terminal sequence of the CP of a HAT isolate of TEV maintained in our laboratory is SGTVDAGADAGKKK ... In this study, the N-terminal sequence of TEV HAT is considered the wild-type for purposes of clarity.

Mutations in the TEV CP were engineered by PCR in a *Sall*-*Bgl*II subclone [pTEV-SB, position 7165 to the 3' end of the poly(A) tail] using a strategy involving the creation of a new *Kpn*I restriction site at position 8520 as described previously (Wang *et al.*, 1996). Briefly, the procedure involved two separate PCR reactions; after adequate restriction of the amplified products both fragments were ligated and inserted into pTEV-SB. Plasmids containing mutations were substituted back into plasmid pTEV-7DA. The series of TEV CP mutants were designated by one letter abbreviations to indicate the changed amino acids and their position from the N terminus of the CP; for instance, pTEV-7DA is referred to as mutant D5G + A8V because it contains these two mutations (see above) with respect to the TEV HAT wild-type sequence.

■ **In vitro transcription, inoculation of plants, and confirmation of mutations.** Purified linearized full-length plasmids were used for *in vitro* transcription in the presence of the appropriate polymerase, nucleotides and the cap analogue m7GpppG (New England Bio-Labs). In the case of TVMV mutants, treatment with Klenow fragment polymerase (Promega) was performed prior to transcription in order to digest the *Sst*I 3' overhang of the linearized plasmid. Transcripts were then obtained by incubation for 1 h at 37 °C in the presence of T7 RNA polymerase (Stratagene) under the conditions recommended by the supplier. For TEV mutants, *Bgl*II-linearized plasmids were incubated for 1 h at 37 °C in the presence of SP6 RNA polymerase (GIBCO-BRL) as indicated by the supplier. Tobacco plants, *Nicotiana tabacum* L. cv. Kentucky 14 (KY 14), were mechanically inoculated with *in vitro* generated transcripts, transferred to a growth room, and the appearance of symptoms was recorded. To confirm the presence of mutations in the virus progeny, samples of infected plants were processed for total nucleic acids extraction, RT-PCR and direct sequencing of the amplified products (Atreya *et al.*, 1991).

■ **Aphid transmission experiments.** Apterous aphids from a colony of *Myzus persicae* Sulz. were collected, starved for at least 2 h in glass vials, and then used in plant-to-plant transmission assays as previously described (Atreya *et al.*, 1990). The amount of virus present in

infected plants used as feeding sources was estimated by serological analysis (ELISA and Western blot) and their relative specific infectivity was determined by mechanical inoculation (dilution assay on tobacco plants in the case of TVMV or local lesion assay on *Chenopodium amaranticolor* plants for TEV). With the exception of the TEV D5K + D9K mutation (see Table 3), none of the mutations had an adverse effect on virus concentration or specific infectivity. KY 14 tobacco plants were used for aphid transmission assays as well as for virus or HC purification.

Virions of mutants selected for *in vitro* transmission studies were purified from transcript-inoculated plants using the procedure of Murphy *et al.* (1990). Purified virions were mixed with TVMV HC (extracted according to Thornbury *et al.*, 1985) for aphid acquisition through Parafilm membranes and transmission to tobacco plants (Pirone & Thornbury, 1983).

If transmission of a mutant occurred, at least one infected plant was chosen at random for sequence analysis, as described above, to confirm the presence of the mutations. For statistical analysis with the SAS package (SAS Institute), each ten-plant experiment was considered a replicate, and the transformation, arc sine of the square root of the proportion of transmission, was applied. The means were separated by the Tukey (HSD) test.

## Results

### Creation of DAL, DAS and DAA motifs in TVMV abolish or reduce aphid transmissibility

The first mutation, substitution of leu for gly, creates the DAL motif found in isolate 3.3 of PPV (López-Moya *et al.*, 1995) and the second, substitution of ser for gly, reproduces the DAS motif found in PSbMV (Johansen *et al.*, 1996). No transmission of the DAL or DAS motif-containing mutants of TVMV was obtained in plant-to-plant transmission tests (Table 1). A previously described DAA motif-containing TVMV mutant (Atreya *et al.*, 1995), which reproduces the motif present in PeMoV, exhibited very low transmission from plant to plant (Table 1).

The transmission efficiencies of mutant and wild-type virions at known concentrations, supplemented with TVMV HC, were compared (Table 2). The mutants with DAS and

DAL motifs were only occasionally transmitted at the highest virion concentration and never transmitted at concentrations of 10 or 1 µg/ml. In the case of the mutant with the DAA motif, the amount of transmission was highly variable between experiments and was consistently reduced two- to tenfold when compared with transmission of wild-type virions (Table 2). The specific infectivity of the mutants, as measured by mechanical inoculation of tobacco with a series of virus concentrations, did not differ from that of the wild-type virus (data not shown).

### Functionality of the DAG motifs in TEV

The HAT isolate of TEV was used to study further the effect of context and to extend our results to another potyvirus. The N-terminal sequence of TEV is similar to that of TVMV, and the HAT isolate has an additional DAG separated from the first by an ala residue (SGTVDAGADAGKKK ...). A series of mutants was created to determine which of these motifs was required for transmissibility. One of the ten mutants (D5K + D9K; Table 3) did not systemically infect tobacco plants (López-Moya & Pirone, 1998) and hence could not be used in transmission tests. The other TEV CP mutants readily infected tobacco plants and had virus titres and specific infectivities similar to wild-type virus. Retention of the mutations in the virus progeny was confirmed as described for the TVMV mutants.

Results of plant-to-plant transmission experiments with the TEV CP mutants are shown in Table 3. TEV HAT was transmitted at a high rate in all experiments. Mutations affecting the first DAG motif or both motifs, such as D5K, G7E and G7E + G11E were not transmitted or were transmitted very inefficiently. Mutations in the second motif alone, such as D9K and G11E, resulted in little or no reduction in transmission. Together, these results implied that the first DAG motif in TEV HAT is the one primarily involved in aphid transmission.

**Table 1.** Effect of substitution of amino acids present in the DAG-like motif of aphid-transmissible potyviruses on aphid transmissibility of TVMV from infected plants

Variant	Virus	N-terminal sequence of CP*	Transmission†	%‡	Range (%)
DAG	TVMV-WT	SDTVDA <b>G</b> KDKARDQK ...	86/90	96 <sup>a</sup>	80–100
DAA§	PeMoV	SDTVDA <b>A</b> KDKARDQK ...	7/120	6 <sup>b</sup>	0–30
DAS	PSbMV	SDTVDA <b>S</b> KDKARDQK ...	0/90	0 <sup>b</sup>	–
DAL	PPV	SDTVDA <b>L</b> KDKARDQK ...	0/90	0 <sup>b</sup>	–

\* Single-letter code is used for amino acids. Bold letters indicate mutations.

† Number of infected plants over number of test plants. Ten aphids per test plant and ten plants per experiment. Results are combined data from all experiments. Each mutant was directly compared with the wild-type virus control in several experiments.

‡ Values followed by the same letter are not significantly different at the 0.05 level according to the Tukey (HSD) test.

§ Includes transmission data from Atreya *et al.* (1995).

**Table 2.** Transmission by aphids of purified TVMV mutants supplemented with purified TVMV HC and acquired through membranes

Aphid feeding solution containing TVMV HC*				
Variant	Virus concentration (µg/ml)	Transmission†	%	Range (%)
TVMV-WT	100	39/40	97	90–100
	10	43/50	86	50–100
	1	21/50	42	30–70
DAA	100	15/20	75	50–100
	10	16/40	40	10–70
	1	8/50	16	10–30
DAS	100	4/40‡	10	0–20
DAL	100	2/60‡	3	0–10

\* Solutions contained purified TVMV mutants at the indicated final concentration and purified TVMV HC.

† Number of infected plants over number of test plants. Ten aphids per test plant and ten plants per experiment. Results are combined data from all experiments. Each mutant was directly compared with the wild-type virus control in several experiments. More than one mutant was tested in each experiment.

‡ 20 test plants per experiment.

**Table 3.** Effect of mutations near the N terminus of the CP on aphid transmissibility of TEV acquired from infected plants

Variant	N-terminal sequence of CP*	Transmission†	%‡	Range (%)
TEV HAT	SGTVDAGADAGKKKD ...	31/40	78 <sup>a</sup>	70–90
D5K	SGTV <b>K</b> AGADAGKKKD ...	0/40	0 <sup>d</sup>	–
D9K	SGTVDAGAK <b>A</b> GKKKD ...	25/40	63 <sup>a,b</sup>	30–90
D5K + D9K	SGTV <b>K</b> AGAK <b>A</b> GKKKD ...	NT	–	–
G7E	SGTVDAEADAGKKKD ...	1/40	3 <sup>d</sup>	0–10
G11E	SGTVDAGADAGEKKD ...	30/40	75 <sup>a</sup>	50–100
G7E + G11E	SGTVDAEADAEKKKD ...	0/40	0 <sup>d</sup>	–
D5G + A8V§	SGTVGAGVDAGKKKD ...	15/40	38 <sup>b,c</sup>	20–60
V4A	SGTADAGADAGKKKD ...	5/40	13 <sup>c,d</sup>	0–20
D5G	SGTVGAGADAGKKKD ...	7/40	18 <sup>c,d</sup>	0–30
G7E + A8V	SGTVDAEVDAGKKKD ...	39/40	98 <sup>a</sup>	90–100

\* Single-letter code is used for amino acids. Bold letters indicate mutations.

† Number of infected plants over number of test plants. Ten aphids per test plant and ten plants per experiment. Results are combined data from all experiments. Each mutant was directly compared with the wild-type virus control in several experiments. More than one mutant was tested in each experiment. NT, Not testable because of lack of infectivity of the transcripts.

‡ Values followed by the same letter are not significantly different at the 0.05 level according to the Tukey (HSD) test.

§ Sequence found in the pTEV-7DA plasmid.

Progeny virus derived from pTEV-7DA was reasonably well transmitted despite having the sequence SGTVGAGVDAGK ...; this contrasts with TVMV, in which the GAG motif resulted in a non-transmissible phenotype (Atreya *et al.*, 1995). To determine whether the other sequence difference between TEV HAT and TEV-7DA (val versus ala in position 8)

also affected its transmissibility, several additional mutants were constructed (Table 3). The mutation D5G resulted in a 50% reduction in transmissibility, while G7E + A8V restored the transmissibility of G7E to wild-type levels, indicating that the presence of a val residue in the position immediately preceding the second motif renders it fully functional for

**Table 4.** Effect of substitutions of the amino acid immediately before the DAG motif on aphid transmissibility of TVMV acquired from infected plants

Variant	N-terminal sequence of CP*	Transmission†	%‡	Range (%)
TVMV-WT	SDTV <b>D</b> AGKDKARDQK ...	45/50	90 <sup>a</sup>	70–100
ADAG	SDT <b>A</b> DAGKDKARDQK ...	34/40	85 <sup>a</sup>	80–90
CDAG	SDT <b>C</b> DAGKDKARDQK ...	32/40	80 <sup>a,b</sup>	70–90
TDAG	SDT <b>T</b> DAGKDKARDQK ...	24/60	40 <sup>b,c</sup>	0–60
SDAG	SDT <b>S</b> DAGKDKARDQK ...	15/60	25 <sup>c</sup>	0–70

\* Single-letter code is used for amino acids. Bold letters indicate mutations.

† Number of infected plants over number of test plants. Ten aphids per test plant and ten plants per experiment. Results are combined data from all experiments. More than one mutant was tested in each experiment.

‡ Values followed by the same letter are not significantly different at the 0.05 level according to the Tukey (HSD) test.

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PPV  ADEREDEEEVDALQPPFVIQAPRRTAPMLNPIFTPATTQPATKPVSVQSGPQLQTFGTYNEDASPSNSNALVNTNRDRDVDAGSGIGFTFVPRL---
TuMV  AGETLDAGLTDEQKQAEKEKEREKAEKERERQRQLALKKGNAAQEEGERDNEVNAGTSGTFSVPRL---
PSbMV  AGDRKKREESIDASQFGSNRDNKKKNKKNKESDTSNKLIVKSDRDVDAGSSGTFITVPRL---
PeMoV  SGDNKSKVEDAAAKLKEKEKEKHKKTEEGTFEGASQTKPEPDVDTCSQGIVNVPRL---
TVMV   SDTVDAGKDKARDQKLADKPTLAIDRTKDKDVNTGTSGTFSIPRL---
TEV    SGTVDAGADACKKKDKDDKVAEQASKDRDVNAGTSGTFSVPRI---

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**Fig. 1.** Amino acid sequence comparison of the N-terminal region of the CP of several potyvirus isolates. Sequences of isolate 3.3 of PPV, a deletion mutant lacking 15 amino acids in the CP (López-Moya *et al.*, 1995), turnip mosaic virus (TuMV; Ohshima *et al.*, 1996), PSbMV (Johansen *et al.*, 1991), PeMoV (Teycheney & Dietzgen, 1994), TVMV (Domier *et al.*, 1986) and TEV (Allison *et al.*, 1986) are aligned with respect to the location of the termini of the trypsin-resistant core (Shukla & Ward, 1989). Residues in bold are those highly conserved among potyviruses. The motifs near the N terminus putatively involved in aphid transmission are boxed on a black background, and the open boxes indicate the position of the motifs located at the beginning of the core.

transmission. Furthermore, mutation of the val located in position 4 to ala in the V4A mutant reduced transmissibility, showing that the position preceding each motif plays an important role in the process.

### Substitutions of the amino acid preceding the DAG motif in TVMV have variable effects on transmission

The effect of the amino acid immediately preceding the DAG was then tested in the TVMV CP. This position is not conserved in potyviruses (Atreya *et al.*, 1991). Since the modification V4A in TEV involved two hydrophobic nonpolar aliphatic residues with only a reduction in size, we tested the same mutation in TVMV as well as three changes to hydrophilic polar uncharged residues. Table 4 shows the results of the transmission experiments performed with these mutants. Mutations of the val to ala or cys, which are smaller and larger than val respectively, did not significantly alter the transmissibility of TVMV. Substitution of ser or thr, hydrophilic but of a size similar to the original val, resulted in a reduction in transmission rates, although the extent of the reduction was much less than that resulting from the val to ala change in TEV (Table 3).

### Creation of a second DAG motif in the CP of TVMV

A DAG motif was identified, by sequence comparison, at the beginning of the CP core of several potyviruses (Fig. 1) including PPV and PSbMV (Maiss *et al.*, 1989; Johansen *et al.*, 1991); in TVMV the motif is NTG. To test the functionality of a DAG motif located in this position in the TVMV CP, this DAG motif was created in the non-transmissible DAL and DAS mutants, resulting in the DAL-DAG and DAS-DAG combination of motifs present in the PPV and PSbMV isolates. No transmission was obtained with these mutants (Table 5). When this second DAG motif was created in wild-type TVMV, resulting in the mutant designated DAG-DAG, the transmission rate was reduced when compared to the wild-type virus (Table 5).

### Effect of a putative complementary motif in TVMV HC

The double mutant was tested to determine whether the presence of a CSC motif in TVMV HC would allow transmission of TVMV that has a DAS motif in the CP. Transcript-inoculated plants were used as the virus source for transmission tests. In two separate experiments, using ten aphids per test plant, no transmission of the CSC-DAG mutant

**Table 5.** Effect of the creation of a second DAG motif near the beginning of the CP core on aphid transmissibility of TVMV

Variant	N-terminal sequence of CP*	Transmission†	%‡	Range (%)
TVMV-WT	SDTVDAGK...DVNTGTS ...	47/50	94 <sup>a</sup>	80–100
DAG-DAG	SDTVDAGK...DVDAGTS ...	15/70	21 <sup>b</sup>	0–40
DAS-DAG	SDTVDASK ...DVDAGTS ...	0/90	0 <sup>c</sup>	–
DAL-DAG	SDTVDALK ...DVDAGTS ...	0/90	0 <sup>c</sup>	–

\* Single-letter code is used for amino acids. Bold letters indicate mutations.

† Number of infected plants over number of test plants. Ten aphids per test plant and ten plants per experiment. Results are combined data from all experiments. More than one mutant was tested in each experiment.

‡ Values followed by the same letter are not significantly different at the 0.05 level according to the Tukey (HSD) test.

occurred to any of 40 total test plants. In contrast, transmission of wild-type TVMV occurred to 18/20 total test plants. The presence of the mutations in progeny virus in the source plants was confirmed as described for the CP mutants, and the mutations were found to have no effect on either virion or HC concentration, as measured by quantitative Western blotting.

## Discussion

Our results indicate that the context in which the DAG motif is located near the N-terminal region of the CP plays a role in determining the efficiency of potyvirus transmission by aphids. Experiments performed with TEV showed that mutations in the first DAG motif of the two present in TEV HAT rendered this virus virtually non-transmissible, while mutations in the second DAG had little effect. However, in certain contexts the second DAG motif of TEV became functional if the first one was altered; the amino acid preceding each motif had a strong effect on transmissibility. In contrast, mutation of the amino acid located before the DAG motif in TVMV did not have the same effect as that observed in TEV. Additional evidence of the importance of the context is that motifs other than DAG, such as DAA, DAL or DAS, present in transmissible isolates of other potyviruses, failed to render TVMV transmissible. The contradictory behaviour of these motifs in their respective viruses and in TVMV indicates that the context surrounding the motif in each virus can affect their function in the transmission process.

In contrast with transmission from infected plants, experiments with purified virus showed that, when supplemented with TVMV HC, the TVMV DAL and DAS mutants were occasionally transmitted and the DAA mutant was transmitted to a considerable extent. The latter situation is similar to that of PPV isolate 3.3, which was not transmitted from infected plants although purified virions could be transmitted when supplemented with HC (López-Moya *et al.*, 1995).

Current evidence suggests that the N-terminal region of the CP needs to interact with the HC for successful transmission. The interaction has been demonstrated in an *in vitro* binding assay, in which point mutations in the DAG motif that abolish or reduce transmissibility also prevent or reduce binding of CP with the HC (Blanc *et al.*, 1997). The N terminus of the CP of potyviruses is surface-exposed on the virion (Shukla & Ward, 1989), allowing the DAG motif to be accessible for interaction with the HC. Mutations in the surrounding residues could produce conformational changes that affect exposure of the motif and thus affect transmissibility. The need for a strong interaction between virions and HC for efficient transmission might explain the discrepancy between our transmission results in plant-to-plant and artificial feeding experiments with the TVMV mutants containing the DAA, DAL and DAS motifs. *In vitro* these mutants are able to interact, although weakly, with the HC (Blanc *et al.*, 1997). Plant cell constituents that might interfere with the CP–HC interaction may not be present in the purified virion/HC mixtures and thus stronger interaction and resultant transmission could occur.

The specificity between a particular virus and its homologous HC is not strict, and several functionally active heterologous combinations have been described, suggesting a broadly conserved mechanism for the transmission of potyviruses by aphids. Functional combinations in which purified HC from plants infected with one potyvirus can assist the transmission of other (but not necessarily all) potyviruses have been reported (Pirone, 1981; Sako & Ogata, 1981; Lecoq & Pitrat, 1985; López-Moya *et al.*, 1995; Flasiński & Cassidy, 1998). However, not all DAG/CP/HC combinations are functional; thus it is possible that the specificity of the process may be affected by sequences in addition to or other than the DAG itself, either in the CP or in the HC. For the CP, this could be the context surrounding the motif. It is important to note that the DAG and positionally equivalent motifs are not

always located at the same positions in the CP N terminus. The DAS motif in PSbMV begins 12 residues from the N terminus, the DAL and DAA motifs in PPV and PeMoV begin at position 11, and in TVMV and the first motif of TEV the DAG motif begins at position 5. The position might affect the accessibility of these motifs and explain why not all HC/CP heterologous combinations are functional. Alternatively, the transmissibility of the PPV and PSbMV isolates with alterations in their primary DAG motifs might be explained by assuming that the secondary DAG motif near the beginning of the conserved CP becomes functional. However, mutations such as DAL-DAG and DAS-DAG, which create this second DAG motif in equivalent positions of the CP of TVMV, did not restore transmissibility of the TVMV DAL and DAS mutants. Interestingly, the control mutant DAG-DAG was transmitted less efficiently than the wild-type, suggesting an adverse effect caused by creation of the second motif. The reduction of transmission observed in the DAG-DAG mutant might be associated with modification of the folding pattern of the N-terminal region of the CP, which could reduce the accessibility of the first motif. At this point our data are not sufficient to conclude if this second motif may be active in other potyviruses.

Our attempt to obtain support for the Flasinski & Cassidy (1998) hypothesis by creating, in TVMV, the DAS CP and CSC HC motifs that are present in the aphid-transmissible PSbMV proved unsuccessful. It should be noted that the context in which this motif lies in the TVMV mutant differs from that in PSbMV; thus the inability of the CSC motif to function in transmission of TVMV might also reflect a requirement of an appropriate context.

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