Mutational analysis of the coat protein N-terminal amino acids involved in potyvirus transmission by aphids

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The nature of the amino acids in the N-terminal ‘DAGX’ motif of the coat protein of tobacco vein mottling virus (TVMV) that have a direct effect on aphid transmissibility of the virion were further defined by site-directed mutagenesis. In the first position of the DAGX motif, Asp or Asn are required for aphid transmissibility. In the second position, the nonpolar residue Ala, but not the nonpolar Gly or Val or the polar Thr and Ser, is compatible with transmissibility. In the third position, the small, neutral, nonpolar Gly appears to be critical; even substitution of Ala, with a minimal side-chain, drastically reduces transmissibility. Although the amino acid following the DAG sequence is not highly conserved among potyviruses, the presence of an acidic Glu or Asp residue at this position in the TVMV coat protein drastically reduces or abolishes aphid transmissibility. An attempt was made to test the hypothesis that trypsin cleavage of the N terminus is involved in the aphid inoculation process by destroying a trypsin cleavage site downstream from the DAGX motif. While the predicted decrease in transmission occurred from infected plants, there was no effect on the transmission of purified virus. Of the 23 mutations in the DAGX region of TVMV reported here and previously, only two, substitutions of Lys and Arg for Asp, had a detectable adverse effect other than on aphid transmissibility. These, and perhaps other, residues near the N terminus function in some phase of the TVMV life cycle, in addition to aphid transmission.

Introduction

Current evidence suggests that successful transmission of potyviruses by their aphid vectors depends upon the interaction of two viral-encoded proteins, the coat protein and the helper component (Pirone, 1991). A triplet of amino acids, Asp–Ala–Gly (DAG), is conserved near the N terminus of aphid-transmissible (AT) potyvirus isolates, and it was speculated that this sequence is required for aphid transmissibility (Harrison & Robinson, 1988). Using site-directed mutagenesis, we demonstrated that certain substitutions in the DAG region result in loss or drastic reduction in aphid transmissibility (Harrison & Robinson, 1988). Comparisons of the effect of these amino acid substitutions in the DAG region of TVMV coat protein with the sequences of other potyviruses (Atreya et al., 1991) suggested the nature of the amino acids at each position that might be conducive for the aphid transmission process. In the first position of the triplet, an acidic or neutral amino acid, but not a basic one, might impart transmissibility. In the second position a small nonpolar residue may be needed and an increase in size or polarity may have an adverse effect on transmissibility. In the third position, Gly seems to be critical and a change to any other residue leads to loss of transmissibility. We therefore targeted mutations to address these hypotheses. Further mutational analysis was also done to determine the effect of deletion of each of the amino acids within the DAG triplet.

The amino acid following the DAG does not appear to be conserved among potyviruses, and it came as a surprise that the change from the wild-type Lys to Glu, done as a control mutation (Atreya et al., 1991), resulted in loss of aphid transmissibility of TVMV. Thus it seemed that the motif involved in aphid transmissibility should be expanded to DAGX, and a mutational analysis of the fourth amino acid was conducted accordingly.

Harrison & Robinson (1988) hypothesized that the DAG sequence might be involved in retention (binding) of virions to the aphid mouthparts, a process that is mediated by helper component (Berger & Pirone, 1986), and that virion release may occur as a result of cleavage of the coat protein N terminus by a trypsin-like enzyme.
samples were electrophoresed on 10% SDS polyacrylamide gels containing 10% SDS, 1% mercaptoethanol and 5% glycerol. The volume of SDS-PAGE sample buffer (50mM-Tri~HC1, pH 6.5 EDTA, pH 8.0) at 37 °C for up to 2 h. At timed intervals, an aliquot of the sample was removed and immediately mixed and boiled with an equal volume of SDS-PAGE sample buffer (50mM-Tris-HCl, pH 6.5 containing 10% SDS, 1% mercaptoethanol and 5% glycerol). The samples were electrophoresed on 10% SDS-polyacrylamide gels followed by immunoblotting using TVMV polyclonal antiserum to detect the virus coat protein and the trypsin-digested fragments.

Methods

The virus isolate TVMV-AT has been described before (Atreya et al., 1992a). The genome-length cDNA clone pXB87, derived from TVMV-AT RNA (Domier et al., 1989) is referred to as pDAGK in this report to indicate the amino acids in the coat protein that were the focus of the present study.

Construction of genome-length cDNA plasmids containing coat protein mutations. Site-directed mutagenesis was done by the method of Kunkel et al. (1987) in the subclone pTVMV-CP, which was constructed as described before (Atreya et al., 1991). The plasmids containing the mutant coat protein genes were identified (Atreya et al., 1990b,c) and subsequently substituted into the genomic-length plasmid pDAGK as described previously (Atreya et al., 1991). The mutations in the full-length plasmids were confirmed by dyeoxygen chain termination sequencing using Sequenase version 2.0 (United States Biochemical Inc.).

In vitro transcription and inoculation of plants with RNA. Transcripts from the linearized plasmid templates containing the T7 promoter were synthesized under the direction of T7 RNA polymerase in the presence of m’GpppG cap analogue; the transcripts were mechanically inoculated to Nicotiana tabacum L. cv. Kentucky 14 (KY 14) seedlings as described before (Atreya et al., 1991). Infected plants were identified by symptom expression as well as by immunodiffusion and immunoblot analysis using TVMV-specific antiserum.

Aphid transmission assays. The general procedures for rearing and handling of aphids and assay plants have been described (Atreya et al., 1990a). Aphids were allowed a 5 to 10 min acquisition access period on infected leaves and then placed on test plants in groups of 10. After an overnight inoculation access period the aphids were killed by spraying with an insecticide, and the plants were placed in a growth room for symptom development.

Confirmation of mutations in viral RNA from infected plants. The presence of the mutation in the progeny viral RNA resulting from mechanical inoculation with the transcripts as well as those infected by aphid inoculation was verified by RT-PCR of viral RNA from the plants, followed by sequencing of the PCR DNA as described before (Atreya et al., 1991).

Quantitative ELISA and infectivity assays. Loss of or reduction in aphid transmissibility can occur as a result of decreased amounts of virus in infected plants. Thus the possible effect of the mutations on the amount of virus in infected plants was tested by ELISA and infectivity tests as described before (Atreya et al., 1991). Each mutant was directly compared with the wild-type virus in both types of test, and at least two separate determinations were made for each mutant.

Trypsin digestion. Wild-type TVMV-AT and the trypsin mutant TVMV-TRP were purified from transcript-inoculated plants and used for in vitro trypsin digestion. In a typical reaction, a virus:trypsin ratio of 200:1 was used in a 100 μl reaction volume (20 mM-Tris, 1 mM-EDTA, pH 8.0) at 37 °C for up to 2 h. At timed intervals, an aliquot of the reaction was removed and immediately mixed and boiled with an equal volume of SDS-PAGE sample buffer (50 mM-Tris-HCl, pH 6.5 containing 10% SDS, 1% mercaptoethanol and 5% glycerol). The samples were electrophoresed on 10% SDS-polyacrylamide gels followed by immunoblotting using TVMV polyclonal antiserum to detect the virus coat protein and the trypsin-digested fragments.

Results

Mutations in the first position of the DAGX motif

Table 1 shows the effect of substitutions in the first position of the DAGX motif on aphid transmissibility of the virus. The wild-type virus has an acidic (Asp) residue in this position, and, as reported earlier, substitution of a neutral residue (Asn) in this position did not adversely affect transmission (Atreya et al., 1991). In the present study, substitution of another acidic amino acid, Glu, or its neutral counterpart, Gln, abolished transmissibility as did substitution of another neutral residue, Gly, or a polar, basic residue, His. We previously reported that the effect of the highly basic residue Lys on transmissibility could not be evaluated, owing to rapid reversion to the wild-type residue (Atreya et al., 1991). Further analysis has shown, however, that the transcript used for inoculation contained a mixture of wild-type and mutant sequences. By re-cloning and selection cDNA was obtained which contained only the Lys mutation, and the transcripts failed to infect either tobacco plants or protoplasts. The presence of another highly basic amino acid, Arg, in this position also resulted in failure to cause a systemic infection, despite a number of trials, and failure to infect protoplasts in a limited number of trials.

Mutations in the second position of the DAGX motif

Table 2 shows the effect of substitutions in the second position of the DAGX motif on aphid transmissibility. In the wild-type TVMV coat protein, Ala, a neutral, nonpolar amino acid is present. Although we had hypothesized (Atreya et al., 1991) that the presence of

Table 1. Effect of substitutions in the first position of the DAGX motif on aphid transmissibility of TVMV

<table>
<thead>
<tr>
<th>Virus</th>
<th>N-Terminal DAGX motif of the CP</th>
<th>Chemical nature of the first residue</th>
<th>Aphid transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Terminal</td>
<td>Acidic</td>
<td>144/160*</td>
<td>90</td>
</tr>
<tr>
<td>Mutants</td>
<td></td>
<td></td>
<td>70-100</td>
</tr>
</tbody>
</table>

* No. of infected plants/no. of test plants. Ten aphids per test plant; ten test plants used in each experiment.

† Mutant from Atreya et al. (1991).

nt, Not testable (because of lack of infectivity).
Table 2. Effect of substitutions in the second position of the DAGX motif on aphid transmissibility of TVMV

<table>
<thead>
<tr>
<th>Virus</th>
<th>DAGX motif of the CP</th>
<th>Chemical nature of the second residue</th>
<th>Aphid transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVMV-AT</td>
<td>DAGK</td>
<td>Neutral, nonpolar</td>
<td>170/180* 94 80-100</td>
</tr>
<tr>
<td>Mutants</td>
<td>T†</td>
<td>Neutral, polar</td>
<td>7/200 3.5 0-20</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>Neutral, polar</td>
<td>0/70 –</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>Neutral, nonpolar</td>
<td>0/70 –</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>Neutral, nonpolar</td>
<td>3/70 4.3 0-20</td>
</tr>
</tbody>
</table>

* No. of infected plants/no. of test plants. Ten aphids per test plant; ten test plants used in each experiment.
† Mutant from Atreya et al. (1991).

Table 3. Effect of substitutions in the fourth position of the DAGX motif on aphid transmissibility of TVMV

<table>
<thead>
<tr>
<th>Virus</th>
<th>DAGX motif of the CP</th>
<th>Chemical nature of the fourth residue</th>
<th>Aphid transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVMV-AT</td>
<td>DAGK</td>
<td>Polar, basic</td>
<td>104/120* 87 70-100</td>
</tr>
<tr>
<td>Mutants</td>
<td>R</td>
<td>Polar, basic</td>
<td>30/30 100 –</td>
</tr>
<tr>
<td></td>
<td>Q</td>
<td>Nonpolar, neutral</td>
<td>24/30 80 70-90</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>Nonpolar, neutral</td>
<td>27/30 90 80-100</td>
</tr>
<tr>
<td></td>
<td>E†</td>
<td>Polar, acidic</td>
<td>1/200 0.5 0-10</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>Polar, acidic</td>
<td>0/30 0 –</td>
</tr>
</tbody>
</table>

* No. of infected plants/no. of test plants. Ten aphids per test plant; ten test plants used in each experiment.
† Mutant from Atreya et al. (1991).

other neutral, nonpolar amino acids might be permissible, substitution of Gly or Val abolished or drastically reduced transmissibility as did substitution of the neutral, polar amino acids Ser or Thr.

Mutations in the third position of the DAGX motif

Loss of aphid transmissibility of potyviruses is most often associated with changes from the Gly found in AT isolates (Table 3 in Atreya et al., 1991). We thus made the minimal change possible, replacing Gly with the slightly larger Ala, which is also neutral and nonpolar. Aphid transmission of the Ala mutant TVMV was reduced to 7% (7/100 test plants infected; range 0–30% transmission) compared to 84% for the wild type (42/50 test plants infected; range 70–100% transmission).

Mutations in the fourth position of the DAGX motif

The effect of substitutions of the amino acid following the DAG triplet on the transmission of the mutant virions by aphids is shown in Table 3. In wild-type TVMV-AT this position happens to be Lys but it is quite variable in many other aphid-transmissible potyviruses. As previously reported (Atreya et al., 1991), aphid transmission of TVMV was drastically reduced when this amino acid was changed to an acidic Glu. In the present study, substitution of another acidic amino acid, Asp, also resulted in loss of aphid transmissibility. On the

Table 4. Deletions in the DAGX motif of the coat protein which result in loss of aphid transmissibility of TVMV*

<table>
<thead>
<tr>
<th>Deletion</th>
<th>Resulting sequence</th>
<th>Resulting change</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAG†</td>
<td>VKDK</td>
<td>Motif lost</td>
</tr>
<tr>
<td>D</td>
<td>VAGK</td>
<td>Loss of Asp or Asn in first position</td>
</tr>
<tr>
<td>A</td>
<td>DGKD</td>
<td>Gly in second position; large residue in third position; Asp in fourth position</td>
</tr>
<tr>
<td>G</td>
<td>DAKD</td>
<td>Large residue in third position; Asp in fourth position</td>
</tr>
<tr>
<td>K</td>
<td>DAGD</td>
<td>Asp in fourth position</td>
</tr>
</tbody>
</table>

* All mutants were infectious via mechanical inoculation and none were aphid transmissible.
† Mutant from Atreya et al. (1991).
other hand, replacement of Lys with another polar, basic amino acid such as Arg, or with neutral amino acids such as Gln or Asn had no effect on the transmissibility of the mutant viruses suggesting that only acidic amino acids at this position have an adverse effect on transmissibility.

**Deletion mutants**

Deletion of the DAG triplet from the coat protein resulted in loss of aphid transmissibility (Atreya et al., 1991). Deletion of each individual residue of the DAG motif had a similar effect (Table 4).

**Mutation of the primary trypsin cleavage site**

Fig. 1(a) shows the primary trypsin cleavage site in the N-terminal domain of TVMV-AT coat protein. Cleavage occurs on the C-terminal side of the Lys which is 15 amino acids downstream from the N terminus (Domier et al., 1986). The effect of mutation of this Lys to Glu is shown in Fig. 1(b), a Western blot comparison of trypsin-treated wild-type and mutant virus. After 2 h treatment with trypsin, a single species of coat protein subunit is seen in the case of wild-type coat protein (lane 2) but in the mutant, trypsin-resistant subunits as well as partially digested coat protein subunits can be seen (lane 4). This suggests that the mutant coat protein is not completely trypsin resistant, probably because the presence of other Lys and Arg amino acids which might be attacked by the enzyme in the absence of the Lys at the preferred position.

Aphid transmission of the trypsin-site mutant, TVMV-TRP, from infected plants was consistently lower than that of wild-type TVMV-AT. In five separate experiments, transmission of TVMV-TRP ranged from 20–30%, compared to 70–100% for TVMV-AT. The difference in transmissibility did not seem to be due to a lower concentration of TVMV-TRP in the plant; relative concentrations of TVMV-TRP and TVMV-AT were very similar when assessed by dilution–infectivity assay (there is no local lesion host for TVMV) and by quantitative ELISA; yields of purified virus were also similar (data not shown).

Aphid transmission was also compared in membrane acquisition experiments, in which aphids were given access to known concentrations of purified virus (Pirone & Thornbury, 1983), in the presence of a standard amount of helper component protein, which is required for transmission of purified potyviruses. In contrast with aphid transmission from plants, there was no difference in the transmission of purified TVMV-TRP and TVMV-AT; transmission for both ranged from 100% at 100 µg/ml or 10 µg/ml to 30% at 100 ng/ml.

**Discussion**

The results described here further define the nature of the amino acids in the N-terminal domain of TVMV coat protein that are compatible with aphid transmissibility of the virion. The requirements for transmissibility are somewhat more flexible than the ‘DAG’ proposed by Harrison & Robinson (1988), but it is clear that only amino acids with particular properties impart transmissibility. Our results also provide further evidence that the presence of a highly basic amino acid in the first position of the DAG has an adverse effect on some critical process in the virus life cycle, other than aphid transmission.

Our previous study (Atreya et al., 1991) showed that substitution of Asn for the acidic Asp in the first position did not adversely affect aphid transmissibility. On this basis we hypothesized that other neutral or acidic residues might be tolerated at this position. However, substitution of the acidic Glu, neutral Gln or Gly, or slightly basic His residues at this position all resulted in the loss of transmissibility (Table 1). We believe that at the first position of the DAGX motif, only Asp or Asn are acceptable for the transmission process to occur, and that the nature or size of the side-chain may be the determining factor. Interestingly, Asn is the only amino acid...
acid other than Asp found in this position in naturally occurring potyvirus isolates (Hammond & Hammond, 1989; Boye et al., 1990; Uyeda et al., 1991).

In our previous study the substitution of Thr for Ala at the second position drastically reduced transmission, suggesting that an increase in polarity at this position is detrimental. This was confirmed by the substitution of Ser which resulted in total loss of transmissibility. However, substitution of other neutral, nonpolar amino acids such as Gly or Val also resulted in loss of transmissibility, suggesting that Ala is specifically required at this position.

The most commonly reported difference between aphid transmissible and nontransmissible isolates is substitution for Gly in the third position of the DAG motif. The presence in this position of acidic residues Asp and Glu, neutral Asn, polar Ser or nonpolar Leu are all associated with lack of aphid transmissibility of a variety of potyviruses (reviewed in Atreya et al., 1991). Thus in the third position, the small, neutral, nonpolar Gly appears to be critical. In the present study, even substitution of Ala, with a minimal side-chain, reduced transmissibility to 6% of the TVMV-AT control. Thus the flexibility offered by lack of a side-chain appears to be necessary at this position.

Although the amino acid following the Asp-Ala-Gly sequence is not highly conserved among potyviruses, we previously found that a Lys to Glu change at this position in the TVMV coat protein drastically reduced aphid transmissibility (Atreya et al., 1991). In the present study (Table 3), aphid transmissibility was lost when this amino acid was mutated to another acidic amino acid, Asp. On the other hand, substitution of a polar, basic amino acid such as Arg or nonpolar, neutral residues such as Gin or Asn had no effect, suggesting that only a negatively charged residue at this position adversely affects aphid transmissibility. Two examples of naturally occurring potyvirus isolates with acidic amino acids in this position are the potato virus Y (PVY) isolates PVY-18 and PVY-D. Lack of aphid transmissibility is associated with the presence of an Asp in this position in PVY-18 (Shukla et al., 1988), whereas PVY-D is reportedly aphid transmissible despite the presence of a Glu in this position (Shukla et al., 1986). In the case of PVY-18 it has not been established that lack of aphid transmissibility is caused by a defect in the coat protein rather than in the helper component protein, while in the case of PVY-D no data were given for the frequency of transmission. Thus, while it is certain that an acidic residue following the DAG adversely affects transmission of TVMV, further studies are needed to determine the importance of this residue for potyviruses in general.

The deletion analyses were initiated in an attempt to assess the importance of each amino acid to virus transmissibility. However, comparison of the resultant sequences in the DAGX position revealed that the deletions had the additional effect of moving certain amino acids into positions that were shown, in the substitution analyses, to be detrimental to aphid transmissibility (Table 4). Thus loss of transmissibility could be ascribed to either the deletion per se or to the presence of an inappropriate residue in that position.

It has been suggested that the DAG sequence is involved in helper component-mediated binding of virions to the aphid mouthparts and that the virion is released for inoculation by the cleavage of the N terminus by trypsin-like enzymes present in the saliva of the aphid (Harrison & Robinson, 1988). Pirone (1991) proposed that the helper component functions either by acting as a ‘bridge’ binding to the DAG region of the virion and to retention sites in the aphid, or by indirectly mediating an interaction between the DAG and the aphid. At present we do not have evidence to distinguish between these alternatives. We attempted to test the release hypothesis by mutation of the Lys that forms the primary trypsin cleavage site downstream of the DAG sequence (Domier et al., 1986) to examine the effect on transmissibility of the mutated virus. While the mutation effectively destroyed this site (Fig. 1b), we found that cleavages still occurred, presumably owing to the presence of other positively charged residues such as Lys and Arg which are still available to trypsin in the mutant. While this might explain the apparent lack of effect of the mutation on transmission of purified TVMV-TRP, it does not explain our observation that aphid transmission is significantly decreased when the TVMV-TRP mutant is acquired by aphids directly from infected leaves. This decrease in transmission of the TVMV-TRP mutant is not due to a lower overall virus concentration in the source plants, since the infectivity and ELISA tests indicated virus concentrations similar to those of TVMV-AT. The difference in transmission of plant-acquired and purified TVMV-TRP might result from a lower level of trypsin-like enzyme activity in the food canal of aphids that acquired the virus from plants, perhaps owing to inhibitors of enzyme activity. The data are insufficient to either support or to refute the proposed hypothesis that virion release is effected by trypsin-like enzymes in the aphid mouthparts. Further mutational analysis of other possible trypsin-recognition amino acids in the N-terminal domain may provide a better understanding of this aspect.

Of the 23 mutations in the DAGX region of TVMV reported here and previously (Atreya et al., 1991) only two had any detectable effect other than on aphid transmissibility; these were the substitutions of Lys and Arg for the wild-type Asp. Neither mutant has infected plants systemically, despite repeated inoculations. They
have also failed to infect protoplasts, in a limited number of tests. It seems more than coincidental that both of these mutations involve changes to highly basic residues, suggesting that the negative effect is exerted at the amino acid level. Furthermore, the fact that neither the deletion nor any of the other substitutions made at this position had such an adverse effect suggests that it is the result of the presence of a deleterious residue at this position, rather than the absence of a necessary residue. These and perhaps other residues near the N terminus function in some phase of the TVMV life cycle, in addition to aphid transmission. Deletion of a part of the N-terminal domain of the tobacco etch virus capsid protein has recently been reported to affect cell-to-cell movement and long distance spread (Dolja et al., 1994), and the TVMV mutants may be similarly dysfunctional.

The authors thank Shari Dutton for technical assistance in virus and helper component purification. Financial support was provided by grants from USDA NRI CGP (91-37303-6505 and 93-37303-9138), USDA ARS Cooperative Agreements 58-6430-1-126 and 58-6430-3-118, and the R.I. Reynolds Corp. J.J.L.-M. is recipient of a Postdoctoral Fellowship from Ministerio de Educaci6n y Ciencia (Spain).

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Virology 178, 161-165.


(Received 5 April 1994; Accepted 18 October 1994)