A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on multiplication

Amit Gal-On, Yeheskel Antignus, Arie Rosner and Benjamin Raccah*

Department of Virology, Agricultural Research Organization, The Volcani Center, P. O. Box 6, Bet Dagan 50-250, Israel

An aphid-transmissible (AT) and two non-aphid-transmissible (NAT) isolates of zucchini yellow mosaic virus (ZYMV) were studied. The predicted amino acid sequences of the coat protein (CP) of the three virus isolates were analysed and compared. The NAT isolates differed from the AT isolate in having a Thr instead of an Ala residue at position 10 in the conserved Asp-Ala-Gly triplet in the N-terminal region of CP.

Aphid transmissibility was restored in a progeny virus derived from an infectious clone of the ZYMV-NAT isolate in which Thr was changed back to Ala by site-directed mutagenesis. However this mutation did not have any effect on the multiplication rate in squash, which was significantly higher than that of the AT isolate. The involvement of this mutation in aphid transmission and virus multiplication is discussed.

Introduction

Zucchini yellow mosaic virus (ZYMV), a member of the potyvirus group, is an important pathogen of cucurbit crops (Lisa et al., 1981). Three isolates of ZYMV differing in aphid transmissibility have previously been characterized (Antignus et al., 1989). The two non-aphid-transmissible (NAT) isolates are present in the host plant at a higher titre than the aphid-transmissible (AT) isolate, as estimated by the number of local lesions on host plants (Antignus et al., 1989).

Lack of transmissibility of potyviruses by aphids may be attributed to either a deficiency in the coat protein (CP) or the production of biologically inactive helper component (HC) (Murant et al., 1988). Harrison & Robinson (1988) compared the amino acid (aa) sequence of the CP gene of several AT and NAT potyviruses, and have proposed the involvement of a conserved triplet of aa [Asp-Ala-Gly (DAG)] located close to the N terminus of CP in AT isolates. The experimental confirmation of this hypothesis became possible with the production of infectious in vitro RNA transcripts of tobacco vein mottling virus (TVMV) (Domier et al., 1989). Atreya et al. (1990) expanded the study by making a series of mutations of that triplet and neighbouring positions, and testing the effect of several aa combinations in that region.

The construction of an infectious full-length cDNA clone (FLC) of ZYMV by Gal-On et al. (1991) has allowed the study of the role of the conserved aa sequence in the N-terminal region of ZYMV CP. It has also allowed the investigation of the possibility that virus multiplication is controlled by the mutation in the DAG triplet of NAT isolates.

Methods

Virus isolates. The three ZYMV isolates used (ZYMV-NAT, ZYMV-NAT* and ZYMV-AT) were those reported in a previous study (Antignus et al., 1989). The NAT-isolates ZYMV-NAT and ZYMV-NAT* were maintained by continuous mechanical inoculation, whereas the ZYMV-AT isolate was propagated by repeated aphid transmission.

cDNA synthesis and cloning. Complementary DNA and cloning of ZYMV-NAT were done as described by Gal-On et al. (1990). cDNA of the 3' end of ZYMV-NAT* and ZYMV-AT RNA was prepared using an oligo(dT) primer with the cDNA Synthesis System Plus (Amer sham). The cloning strategy was based on sequence data for the ZYMV-NAT CP gene (Gal-On et al., 1990). The cDNAs were digested with XhoI and ligated into a XhoI-cleaved Bluescript vector (KS) (Stratagene). Clones representing 1374 nucleotides (nt) from the 3' end of the viral gene (including 879 nt of the CP gene) were obtained and named pZY-ATcp and pZY-NAT*$cp.

Nucleotide sequencing. Clone ZYKS-22cp, representing the CP gene of ZYMV-NAT, was cloned and sequenced by Gal-On et al. (1990). This clone is designated herein pZY-NATcp. Sequencing of both strands of pZY-ATcp was carried out as described by Gal-On et al. (1990). DNA sequencing was based on the dideoxynucleotide chain termination method (Sanger et al., 1977), using T7 DNA polymerase (Sequenase; US Biochemicals). Clone pZY-NAT*$cp was sequenced as above to identify the first 180 nt at the 5' end of the CP gene.

Site-directed mutagenesis. Clone pZY-NATcp served as a template for oligonucleotide-directed mutagenesis. Single-stranded DNA was obtained from Escherichia coli strain CJ236 transformed with the...
pZY-NATcp plasmid and superinfected with the helper phage R408. Mutagenesis was performed according to the method of Kunkel et al. (1987) using the oligonucleotide 5' GCAGAC G CTGAG 3'. The sequence of this oligonucleotide is the same as that of the NAT isolate CP gene nt 22 to 34 with a G residue instead of an A residue at position 28 (underlined), the target for mutagenesis (Fig. 2). The resulting mutant clone, in which a G residue replaces the A residue of the NAT isolate, was named pZY-NAT't; it contains the whole ZYMV-NAT CP gene, but encodes Ala instead of Thr at aa position 10. The presence of the mutation of the NAT CP gene sequence (G instead of A) was confirmed by sequencing the region.

Construction of the mutated FLC. Construction of the FLC of ZYMV-NAT (pKSM16322M) from three different clones has been described in Gal-On et al. (1991). For simplicity, this clone will be designated herein as pZYFLC-NAT. The three clones (ZYKS16, ZYKS3 and ZYKS22) forming the FLC of ZYMV-NAT (Gal-On et al., 1991) will be designated pZY16, pZY3 and pZY22. The construction of a mutated FLC required the exchange of the original Xhol fragment with the mutated Xhol fragment from clone pZY-NAT'tcp (Fig. 1). This was carried out in three steps. (i) The strategy for exchanging the regions carrying the CP gene was based on digestion with Xhol at the two sites flanking the CP gene region. The presence of a third Xhol site in the FLC, external to the region to be exchanged, was required to prevent unwanted cleavage. This was achieved by PstI digestion, thus producing the pZY3 fragment. The PstI linearized construct with two unique PstI sites was religated producing clone pZY16+22 (Fig. 1a). (ii) The region between the Xhol sites in pZY16+22 was interchanged with the mutated Xhol fragment from pZY-NAT'tcp, thus producing clone pZY16+22' (the '1' designation denotes the incorporation of a mutation in the CP gene) (Fig. 1b). The correct orientation of the inserted clone was verified by locating the Xhol site at the 3' end of the non-coding region (Gal-On et al., 1990). (iii) The FLC was reconstructed by reinsertion of clone pZY3 into the PstI site of clone pZY16+22'. The mutated pZYFLC-NAT is named pZYFLC-NAT't (Fig. 1c). The correct orientation of the insert fragment was verified by determining the position of the third Xhol site in pZYFLC-NAT't (Fig. 1).

In vitro transcription. In vitro transcription was carried out as described in Gal-On et al. (1991). The transcription reaction contained 40 mM-Tris-HCl pH 7.5, 50 mM-NaCl, 8 mM-MgCl2, 2 mM-spermidine, 20 mM-DTT, 0.5 mM-ATP, 0.5 mM-CTP, 0.5 mM-UTP, 0.05 mM-GTP, 1 mM-5'GpppG (New England Biolabs), 20 units/µl T7 RNA polymerase (Stratagene), 100 ng/µl linearized FLC (pZYFLC-NAT and pZYFLC-NAT't) (Table 1) and 1 unit/µl RNasin (Promega).

Plant inoculation. In vitro capped RNA transcripts in their original reaction mixtures were diluted 1:1 with double-distilled (dd) H2O and immediately used for inoculation of squash seedlings. The cotyledon surface was dusted with carborundum and each plant was mechanically inoculated with 70 µl of transcription reaction mixture (about 6 to 8 µg RNA). Control seedlings were inoculated with either transcription buffer or with 0.5 µg ZYMV RNA dissolved in 20 µl of ddH2O per plant (the amount of RNA was estimated by ethidium bromide staining of an agarose gel). Aphid inoculation was carried out as described in Antignus et al. (1989). Three Myzus persicae were used for each squash test plant. Acquisition access feeding was for 10 min and inoculation access feeding for 2 h.

Determination of virus titres and accumulation in plants. ZYMV was purified as described in Antignus et al. (1989). The virus titre in plants infected with ZYMV-NAT or ZYMV-AT was determined in three separate experiments. An additional experiment was made to compare ZYMV-NAT and ZYMV-AT with ZYMV-NAT't (virus derived from the RNA transcripts of pZYFLC-NAT't). Parallel mechanical inoculation of squash seedlings took place at the same time, applying 20 µl of purified ZYMV-AT, ZYMV-NAT or ZYMV-NAT't at a concentration of 0.5 µg/ml to each plant. Parallel sampling of virus was made at the same time after inoculation for each virus isolate. A pair of disks (1 cm diameter) was removed from between eight and 12 infected plants for each isolate. Samples were kept under refrigeration until the last sampling (3 or 4 weeks) was completed. The samples were homogenized in ELISA extraction buffer (0.5 ml/disk) (Clark & Adams, 1977), and the resulting homogenate was diluted 1:5 and applied in duplicate to Dynatech microtitre plates. This procedure ensured that virus determination in samples taken at different times was made in the same ELISA plates. Antiserum against ZYMV (IgG) and an alkaline phosphatase conjugate were diluted 1:1500. Absorbance at 405 nm (A405) was determined using a model EL310 Bio-Tek reader. Data were compared by analysis of variance.

Results

Comparison of the nucleotide and the predicted aa sequence of the CP genes of AT and NAT isolates of ZYMV

Comparison of the CP genes of NAT and AT isolates was aimed at detecting differences in regions related to aphid transmissibility. At the nucleotide level, 99% identity was found between the ZYMV-NAT (Gal-On et al., 1990) and the ZYMV-AT CP genes. Comparison of the nucleotide sequence of the genome region encoding the CP core of ZYMV-NAT (Gal-On et al., 1990) with that of ZYMV-AT (data not shown) revealed three silent
nucleotide transitions at positions 600, 657 and 710. Two transitions were found in the 5' end of the CP gene; one, from G to A, resulted in a change from Ala to Thr (within the Asp-Ala-Gly triplet) at position 10 and another, from A to G, resulted in a change from Ser to Gly at position 33 in the N terminus of the ZYMV-NAT CP (Fig. 2). Comparing the aa sequence in the N-terminal region of the CP of the second NAT isolate (ZYMV-NATmv) with
Table 1. Infectivity and transmissibility of in vitro RNA transcripts of the FLCs tested on squash plants

<table>
<thead>
<tr>
<th>RNA inoculated</th>
<th>Mechanical inoculation infected/tested (%)</th>
<th>Aphid transmissibility* infected/tested (%)</th>
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<tbody>
<tr>
<td>Mock inoculation†</td>
<td>0/5 (0)</td>
<td></td>
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<tr>
<td>Capped RNA transcript</td>
<td></td>
<td></td>
</tr>
<tr>
<td>of pZYFLC-NAT†</td>
<td>1/10 (10)</td>
<td>0/35 (0)</td>
</tr>
<tr>
<td>Capped RNA transcript</td>
<td></td>
<td></td>
</tr>
<tr>
<td>of pZYFLC-NAT§</td>
<td>19/33 (58)</td>
<td>42/47 (89)</td>
</tr>
<tr>
<td>Wild-type ZYMV-AT RNA</td>
<td>8/8 (100)</td>
<td>18/22 (82)</td>
</tr>
<tr>
<td>Wild-type ZYMV-NAT RNA</td>
<td>5/5 (100)</td>
<td>0/35 (0)</td>
</tr>
</tbody>
</table>

* Aphid transmissibility was assayed using three M. persicæ/test plant.
† Mock inoculation was done using the linearized clone with the transcription reaction components but without T7 RNA polymerase.
‡ pZYFLC-NAT represents the FLC of the NAT isolate of ZYMV (ZYMV-NAT).
§ pZYFLC-NAT§ is the FLC with a mutation in the genome region encoding the N-terminal region of the wild-type ZYMV-NAT CP.
‖ The infectivity of the capped pFLC-NAT† RNA transcripts represent the sum of three separate transcription reactions. The infectivity of each was 6/11, 10/19 and 3/3.

that of ZYMV-AT revealed two mutations; one was specific for ZYMV-NATmy and resulted in a change from Pro to Gln at position 5 (mutation of nt 14); the other changed Ala to Thr at position 10 in the predicted aa sequence of CP (Fig. 2).

Involvement of the Asp-Ala-Gly triplet in aphid transmissibility of ZYMV

Mutated infectious RNA transcripts were made to determine whether transmissibility of ZYMV-NAT could be restored by changing the Asp-Thr-Gly triplet to Asp-Ala-Gly of the AT isolate. As shown in Table 1, 58% (19/33) of the squash seedlings mechanically inoculated with the in vitro RNA transcripts of pZYFLC-NAT† were infected and produced typical ZYMV symptoms. The original non-mutated pZYFLC-NAT RNA transcripts were infectious in only 10% (1/10) of seedlings. Controls included mechanical inoculation with wild-type ZYMV-NAT and ZYMV-AT RNAs, and resulted in 100% infection (8/8 and 5/5). The aphid transmissibility rate of the progeny virus derived from pZYFLC-NAT† RNA transcripts was 89% (42/47) (Table 1). This rate was similar to that obtained with aphids fed on seedlings infected with virus from the wild-type ZYMV-AT RNA (18/22, 82%). No transmission (0/35) was obtained either in the seedlings infected with virus originating from pZYFLC-NAT transcripts or in seedlings infected with wild-type ZYMV-NAT RNA (Table 1).

Multiplication rate of the ZYMV isolates in infected squash plants

The specific infectivity of purified preparations of ZYMV-NAT and ZYMV-AT (expressed as the number of local lesions produced on Chenopodium amaranticolor plants) was compared and found to be about the same (data not shown). Purification of the ZYMV-NAT isolate always yielded more virus per leaf weight as compared to ZYMV-AT. The rate of multiplication (as reflected by the level of the virus CP in the host plant) was quantitatively estimated and compared by ELISA. Multiplication of ZYMV-NAT in squash plants was significantly higher than that of ZYMV-AT (Fig. 3a, b, c). ELISA values for plants inoculated with the virus progeny derived from pZYFLC-NAT† RNA transcripts are given in Fig. 3(d). Both ZYMV-NAT† (virus derived from the RNA transcripts of pZYFLC-NAT†) and the wild-type ZYMV-NAT isolates accumulated to a similar level, whereas the ZYMV-AT isolate had a significantly lower rate of multiplication. The possibility that differences in the levels of CP between the ZYMV-NAT and ZYMV-AT isolates were a result of the level of expression of CP was eliminated by monitoring viral RNA accumulation by hybridization. Significantly higher levels were recorded for ZYMV-NAT RNA than for ZYMV-AT RNA (data not shown).

Discussion

A nucleotide transition resulting in an aa mutation from Ala (in AT) to Thr (in NAT and NATmy) was found in the Asp-Ala-Gly triplet. This finding is different from those for other NAT potyviruses for which the common mutation in this triplet was from Gly to Asp, Glu, Gln, Ser or Leu (Atreya et al., 1991). Direct proof for the role of the triplet in ZYMV aphid transmissibility was obtained by creating a mutation from Thr to Ala in the N terminus of CP, thus restoring aphid transmissibility. Our study provides the first confirmation of the findings of Atreya et al. (1990) on the association of the Asp-Ala-Gly triplet of TVMV CP with aphid transmissibility in another potyvirus.

Atreya et al. (1991) made a mutation changing Ala to Thr in the Asp-Ala-Gly triplet of TVMV (based on our ZYMV-NAT data) which strongly reduces but does not abolish aphid transmissibility. This finding led them to suggest that residual transmissibility may also exist in ZYMV-NAT. However, in thousands of attempts carried out both in our laboratory and in Montfavet, France, we have never succeeded in transmitting ZYMV-NAT using aphids (data not shown). The difference between TVMV and ZYMV may imply an
importance of the context of the protein sequence within which the Asp-Ala-Gly triplet lies. However, it is evident that both the second and the third amino acids in the Asp-Ala-Gly triplet are essential for aphid transmissibility, although the extent of aphid transmission loss may depend on the nature of the potyviral HC, CP and/or the type of host plant involved.

The role of various aa changes in the conserved triplet and the lysine adjacent to it has been thoroughly investigated by Atreya et al. (1991). However, direct experimental data explaining the mechanism of aphid transmissibility involving aa in the N-terminal region are lacking. We hereby propose two hypotheses based on the data of Atreya et al. (1991) and on the present study. (i) The aa in the Asp-Ala-Gly serve as a binding site, either for HC or the aphid's stylets. (ii) The aa in the triplet are not directly involved in binding, but affect the folding of the N-terminal region in a manner that exposes (in AT isolates) or covers (in NAT isolates) a binding site that may be located in another site in CP. Conformational changes are anticipated when the so-called neutral Gly is replaced by charged, hydrophilic or hydrophobic amino acids. In the NAT isolates, Gly is replaced by a charged aa [Gly to Glu in TVMV (Atreya et al., 1990); Gly to Asp in soybean mosaic virus (Jayaram et al., 1989)] or by a hydrophobic aa [Gly to Leu in plum pox virus (Maiss et al., 1989)]. The two explanations suggested above may also hold true for ZYMV, but testing them will require the insertion of additional mutations and deletions in CP and a better understanding of the relationships between CP and HC.

The association between aphid transmissibility and virus multiplication in the host has also been reported by others [e.g. for the potyvirus TEV (Simons, 1976) and pea enation mosaic virus (Hull, 1977)]. The fact that ZYMV-NAT accumulates in the host plant to a higher level than the AT isolate raises the possibility that transmission and virus multiplication are jointly controlled. However, the mutated ZYMV-NAT produces AT virions, but multiplies at the same high rate as its ZYMV-NAT ancestor, thus indicating that the control of virus multiplication is not linked to the mutation that restored aphid transmissibility. However, the question whether the relationship between lack of aphid transmissibility and elevated virus titre in the host plant is casual or whether both properties, though unrelated, are selected under similar growth conditions remains open.

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References


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