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BARD

FINAL REPORT
PROJECT NO. US-2088-91

**The Role of Specific Viral Genes and Gene
Products in Potyviral Pathogenicity, Host
Range and Aphid Transmission**

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1996

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הספריה המרכזית
למדעי החקלאות
בית דגן

Date: June 17, 1996

BARD Project Number: US-2088-91RC

Project Title:

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Pathogenicity, Host Range, and Aphid Transmission

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Project Start Date: 8/15/92

Type of Report: 1st Annual _____ 2nd Annual _____ Final X

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Authorizing Official, Affiliated Institution 6-19-96

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ABSTRACT

Tobacco vein mottling virus (TVMV) and zucchini yellow mosaic virus (ZYMV) are two members of the plant virus family *Potyviridae* that have different hosts. To attempt to reveal features of these viruses that may be determinants of host range, symptom expression and aphid transmissibility, we have used recombinant DNA techniques to exchange parts of the genomes of these viruses and tested the effects of the chimeric and mutant viruses in infectivity and transmission assays. In addition, several series of site-specific mutations have been made in coding regions of both viral genomes and in that of tobacco etch virus (TEV).

The experiments have resulted in a detailed description of specific regions in potyviral genomes that play a role in the transmission of viruses from plant to plant by aphids. Essential residues in the HCpro coding region have been identified and the amino acid sequence motif near the N-terminus of potyviral coat proteins has been defined in considerable detail.

Sequence changes in various regions in the genomes of the potyviruses that were investigated have been shown to affect viability and symptom expression. The most striking example of an effect on symptoms was the production of a wilting response in infected pepper plants when a segment in a non-wilting strain of TEV was replaced by the equivalent segment from the wild type (wilt-inducing) virus. No region has been shown to be a direct determinant of viral host range. Some changes in amino acid sequence in the VPg-NIa and coat proteins of TVMV had an effect on the ability of the virus to spread systemically in plants.

OBJECTIVES OF ORIGINAL PROPOSAL

The aim of the proposal was to increase our understanding of the mechanisms that determine the host range, expression of symptoms and aphid transmission of potyviruses. The original, specific objectives were:

1. Identification of amino acid sequences in potyviral coat proteins that affect symptom expression and host range.
2. Determination of the requirements for aphid transmission of other potyviruses of an amino acid sequence motif, asp-ala-gly (DAG), in the coat protein of tobacco vein mottling virus (TVMV) that has been shown to be required for transmission.
3. Determination of the role of the HCpro coding region in potyvirus genomes in symptom expression, host range and aphid transmission.

In the course of these studies, we have also examined the effects of alterations in other coding regions of potyviral genomes, and in the 3'-untranslated region of TVMV RNA.

METHODS

Most of the work in this project has been done with three potyviruses, TVMV, zucchini yellow mosaic virus (ZYMV) and tobacco etch virus (TEV). The main approach has been the introduction, by standard *in vitro* mutagenesis techniques, of specific alterations in the various coding and noncoding regions of these viruses, and the determination of the effects of such changes on the phenotype of the viruses. Particular emphasis has been placed on changes in the HCpro and coat protein coding regions.

Four different plant species, *Nicotiana tabacum* (tobacco), *N. benthamiana*, *Cucurbita pepo* (zucchini) and *Capsicum frutescens* cv. Tabasco have been used to

assess changes in host specificities of the variant forms of the viruses. Symptom expression and aphid transmission assays have been performed by well-established methods.

RESULTS

Sequence Alterations That Affect Symptom Expression

Changing a lys residue in the N-terminal part of the TVMV HCpro coding region (at position 51) to a glu residue resulted in an attenuated symptom phenotype and reduced accumulation of virus in infected plants. Similar results were obtained when the lys was changed to a his or a gln residue. A mutant in which the cys residue at position 35 had been changed to a ser residue also gave rise to the attenuated phenotype. The substitution of ser for cys at positions 22, 24 and 54 resulted in no detectable systemic infection up to 5-6 weeks post inoculation. In the case of the mutations at positions 24 and 54, symptoms appeared after 5-6 weeks but sequencing revealed the presence of wild-type RNA. Thus, sufficient replication of the mutant RNA had taken place to allow reversions to occur (Atreya and Pirone, 1993).

A mutant of TVMV in which a four-amino acid segment had been introduced into the C-terminal part of the HCpro protein produced a cyclical pattern of symptoms in infected tobacco plants (Klein *et al.*, 1994). Leaves with no or a few, scattered chlorotic areas were followed by those that developed typical vein mottling symptoms and, as these alternating effects were repeated as the plants continued to grow. A similar phenotype was produced by a similar insertion in the P1 protein of TVMV.

The determinants in a strain of TEV (HAT) that causes root necrosis and wilting in Tabasco pepper plants were investigated by exchanging segments of its

RNA genome with those in a non-wilting strain of the virus (NW) and testing the effects on symptom production by the chimeric RNAs (M.-H. Chu, Ph.D. dissertation, University of Kentucky). Substitution of the P1, HCpro, P3, NIb or coat protein coding regions of TEV HAT RNA by those in TEV NW RNA did not diminish the wilting response in inoculated plants. However, replacement of a segment of TEV NW RNA containing part of the CI and the full 6K coding regions with a similar segment from TEV HAT RNA resulted in a virus that produced the wilting phenotype. Another wilting determinant was identified in a segment of TEV HAT RNA that contains the P3 and part of the NIa coding regions.

A mutant of TVMV, in which the three amino acid residues at the C-terminus of the coat protein (lys-gly-val) were changed to the substituted by four residues in the same position in the coat protein of ZYMV (asn-thr-met-gln), was constructed. *N. benthamiana* plants inoculated with the mutant produced mild vein clearing symptoms rather than the severe leaf distortion displayed by TVMV-inoculated plants.

A variant of TVMV that contains four extra, tandemly arranged copies of a AU-rich, 14-nucleotide sequence segment that is present in the 3'-untranslated region of the wild type RNA, is able to infect and spread in inoculated tobacco plants but does not cause symptoms. One copy of the segment can be removed without affecting symptom attenuation. Substitution of the extra copies by AU-rich mRNA instability factors does not mimic the phenotype. There is evidence of the cross-protection effect in variant-infected plants that are subsequently inoculated with wild type TVMV (Qiu and Shaw, 1996).

In all these mutagenesis studies, we have produced many mutants that do not produce symptoms because they are not viable, presumably as a result of deleterious effects on replication of RNA.

Sequence Alterations That Affect Aphid Transmission

A detailed mutational analysis of the HCpro coding region of TVMV and its role in aphid transmission has been conducted. Changing the lysine residue at position 51 to a glu residue resulted in a loss of aphid transmissibility of the virus (Atreya *et al.*, 1992). Other mutants in which residue 51 was changed to a gln or his residue were also deleterious to transmissibility but substitution by an arginine residue was not (Atreya and Pirone, 1993). HCpro isolated from plants infected with a mutant in which a cys residue at position 35 had been changed to a ser residue was unable to effect transmission of virus by aphids.

There has been continued investigation of the "DAGX" motif that is located near the N-termini of potyviral coat proteins and has been shown to be involved in aphid transmission of potyviruses. In the first position of the motif, either an asp or an asn residue was shown to be necessary for transmission of TVMV; in the second, substitution of the ala residue by gly, val, thr or ser residues was deleterious; changing the gly residue in the third position to ala seriously reduced transmission (Atreya *et al.*, 1995). Changing the amino acid in the fourth position to glu or asp was also deleterious to transmission.

The context in which the motif is located has also been investigated using the HAT isolate of TEV which has the sequence SGTVDAGADAG near the N-terminus of the coat protein (López-Moya and Pirone, 1996). Alteration of the first DAG resulted in loss of aphid transmissibility; thus, in this context, the second DAG is not

functional for transmission. However, mutation of the ala residue between the two DAG motifs to val rendered the second DAG functional.

Nucleotide sequence comparisons between transmissible and nontransmissible strains of ZYMV have revealed three differences in amino acid sequence in the central region of the HCpro coding region (Huet *et al.*, 1994). Substitution of two of these in the transmissible strain by those in the nontransmissible strain (asp148 to gly and arg180 to ile) resulted in reduced transmissibility; changing thr309 in the conserved "PTK" motif to ala caused almost total loss of transmissibility. Mutants in which thr309 has been changed to a ser or a val residue had greatly reduced helper activity. Western blot analysis suggested that the level of HCpro in plants infected with the mutants was about the same as in plants infected with wild-type virus.

Sequence Alterations That Affect Movement of Virus in Infected Plants

Charge-specific alterations in the DAG sequence in the coat proteins of both TVMV and TEV were shown to affect cell-to-cell movement of the viruses (López-Moya and Pirone, 1996). Mutation of the asp residue to lys or arg in TVMV resulted in lack of systemic infection but did not affect replication in protoplasts. In the case of TEV HAT, which has two N-terminal DAG motifs, substitution of lys for both asp residues was required to stop systemic infection.

The S strain of TVMV is able to move from cell to cell and throughout the plant in the TN 86 cultivar of burley tobacco, which is resistant to systemic infection by wild-type TVMV. A DNA copy of TVMV-S RNA has been constructed and cloned and it has been shown that differences in sequences in the VPg coding region are correlated with the differential intercellular movement of TVMV and TVMV-S in TN 86 tobacco (Nicolas *et al.*, 1996).

Construction of TVMV-ZYMV Chimeric Viruses

The HCpro coding region in TVMV was replaced with that of ZYMV and the chimeric nature of the resulting virus was verified by PCR and sequence analysis (Atreya and Pirone, 1993). The chimeric virus could infect *N. benthamiana* plants but the symptoms that developed in the plants were less severe than those produced in TVMV-infected plants. Direct inoculation of tobacco plants with chimeric transcripts did not result in infections though virus from transcript-inoculated *N. benthamiana* plants did infect tobacco plants. In infected tobacco plants, the chimeric virus produced an attenuated symptom phenotype and a reduced accumulation of virus. Normal proteolytic processing of HCpro occurred in chimera-infected *N. benthamiana* plants and active HCpro could be isolated from the plants.

We produced a number of chimeric constructs in which all or parts of the coat protein coding region of TVMV or ZYMV were replaced by equivalent parts of the coat protein coding region of the other virus:

CHIM1: entire coat protein coding region in TVMV replaced by that in ZYMV; all up- and downstream TVMV sequences retained;

CHIM2: same as CHIM1 except first four amino acid residues of coat protein coding region were those of TVMV (SDTV) instead of ZYMV (SGTQ);

CHIM3: same as CHIM1 except N-terminal 40 amino acid residues were those of TVMV;

CHIM4: same as TVMV except C-terminal 3 residues (KGV) replaced by C-terminal 4 residues of ZYMV (NTMQ);

ZYCPTV: segment of ZYMV genome between 11th codon of ZYMV coat protein gene and 15th nucleotide downstream from ZYMV polyprotein stop codon replaced by TVMV coat protein coding region.

Each of these chimeric constructs was inserted into the appropriate full-length cloned DNA (TVMV for the CHIM series and ZYMV for ZYCPTV) and transcripts were tested for infectivity in protoplasts and plants. CHIM1, CHIM2 and CHIM3 transcripts did not give rise to detectable infections. CHIM4 transcripts appear to be able to infect *N. benthamiana* plants and are currently being further tested. Neither ZYCPTV transcripts nor DNA constructs containing the 35S promoter (see below) were able to cause detectable infections in tobacco, *N. benthamiana* or zucchini plants. On the other hand, ZYCPTV transcripts occasionally produced local lesions on leaves of *Chenopodium amaranticolor* plants, but virus could not be recovered from these lesions nor could extracts of the lesions promote infections in other plants.

The ability of each of the chimeras to effect proteolytic processing at the NIb-CP cleavage site was tested. Subclones containing the NIa, NIb and coat protein coding regions were constructed and transcribed *in vitro* and the transcripts were used in the wheat germ lysate (CHIM series) or rabbit reticulocyte lysate (ZYCPTV) translation system. In each case, processing occurred, including that where the amino acid sequence immediately downstream from the cleavage site had been altered (CHIM1).

Construction of a fifth chimera in the TVMV CHIM series is nearing completion. Its coat protein coding region consists of the first 26 codons of the TVMV coat protein coding region followed by all but the first 40 codons of ZYMV coat protein coding region.

In the course of these studies, an improved system for generating infections with ZYMV constructs and for applying them to plants has been developed (Gal-On *et al.*, 1995). Mechanical inoculation of the full-length clone of ZYMV that was placed under the control of the CaMV 35S promoter resulted in twice the efficiency of inoculation compared to RNA transcribed from the T7 promoter-containing clone. However, the use of the particle bombardment method for inoculation increased the efficiency almost 10^6 -fold compared to mechanical inoculation. Uncapped transcripts, and transcripts with extra nucleotides that were not infectious when applied to plants mechanically, produced infections when applied by the bombardment method.

DISCUSSION AND CONCLUSIONS

Whether or not a particular RNA virus will infect and cause a disease in a given plant is dependent upon whether it can infect and replicate in one or more cells of the plant and can then spread to other cells and tissues. The host range of a virus consists of those species of plants in which inoculation results in the occurrence of these events. Thus a host range determinant is probably equivalent to a determinant of replicability of the viral genome or a determinant of intercellular movement of virus (particles or viral nucleic acid).

The results of our investigations are consistent with the notion that there are no direct genetic determinants of host range in plant viruses. None of the mutants we have constructed in the course of these studies has been unequivocally shown to infect a plant species that could not be infected by the virus from which it was constructed. Nor were any of them unable to infect a type of plant that is susceptible to the parent virus. On the other hand, a number of mutants had adverse

effects on replication of viral RNA and a few may have movement-defective phenotypes.

The most significant results of our studies come from the analyses of aphid transmissibility determinants in the HCpro protein and coat protein of potyviruses. They have provided the primary structure information upon which are based both the structural constraints and degrees of latitude that govern the vital interaction between the two proteins. The array of mutants will be invaluable when it becomes possible to examine the structures of the two proteins (and of the coat protein in the virion form) by X-ray diffraction analysis. In the meantime, they will provide excellent subjects for biochemical studies (such as in the yeast two-hybrid system) of their interactions.

COOPERATIVE ARRANGEMENTS

Clones containing parts of the TVMV and ZYMV genomes were exchanged between the University of Kentucky and Volcani Centre laboratories so that the chimeric viruses could be prepared.

There was a ready exchange of information concerning nucleotide sequence analyses, insect transmission assays, protoplast inoculation procedures, and other experimental protocols. Some of this information was required for the preparation of mutant viruses and some was provided when one or the other laboratory sought expert advice on the utilization of a new or refined experimental procedure.

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