Replication and Long Distance Transport of Tobacco Etch Virus in Plants Dependent upon Helper Component-Protease

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INTRODUCTION

Potyviruses belong to the picornavirus-like supergroup of positive-strand RNA viruses [6]. Potyviruses form the largest and most economically important group of plant viruses [7]. This group includes such viruses as potato Y virus, tobacco vein mottling virus, plum pox virus, and tobacco etch virus (TEV), which is the model system used for this research. Potyviruses have a filamentous structure consisting of numerous capsid proteins surrounding a single molcule of RNA with a virus encoded protein attached to 5' end. The single-stranded RNA of 10,000 nucleotides encodes a single polyprotein which autocatalytically cleaves itself into nine proteins. These nine proteins provide the means by which the virus replicates in infected cells, moves to neighboring cells, and moves to other parts of the plant through the vascular tissue of the plant. Among the viral proteins are three proteases P1, HC-Pro, and NIa. One of these, HC-Pro, is the focus of this study.

HC-Pro (Helper component-protease) protein, which aggregates as amorphous inclusion bodies within the cytoplasm of infected plant cells [4], has at least two functional domains and possibly a third. The carboxy terminal end of the protein contains the protease domain. This protease function cleaves the carboxy terminus of HC-Pro from the neighboring P3 protein which has not yet been assigned a function. The amino terminal helper component domain, in particular lysine 54, allows the virus to be transmitted from plant to plant via aphids [11]. This domain is not needed for viral replication

or movement between plant cells, although deletions of this domain greatly decrease the efficiency of viral replication [7]. This decrease in replication efficiency suggests that HC-Pro may function as an accessory factor in replication. The presence of a group of conserved cysteine residues suggests the possibility of a metal binding motif analogous to the zinc finger motif of several eukaryotic transcription factors [12,]. In addition to the two terminal domains, the central part of the protein may constitute a third functional domain. This region, although well conserved in potyviruses, is absent in related viruses such as barley yellow mosaic virus (BaYMV) and the chestnut blight hypovirulence-associated double stranded RNA (HAV) [8]. In conjunction with the N-terminal domain of HC-Pro, this central region may function as an accessory factor to the viral replication complex.

To investigate this possibility, five highly conserved sites were identified within the area by comparison to the amino acid sequences of related potyviruses. Two of these sites (coding for three amino acids each) were selected for mutagenesis. Here the preliminary characterization of the replication and movement characteristics of these mutants are reported.

MATERIALS AND METHODS

To aid characterization of the mutant viruses an engineered virus (TEV-G↓HC) containing a gene coding for the reporter protein β-glucuronidase (GUS) was used instead of the wild-type TEV. This reporter protein, which cleaves flourometric and colorimetric substrates, allowed for simple assays of

virus movement and replication.

Generation of the mutations used in this experiment required the use of a small plasmid containing DNA corresponding to the nucleotide sequence between nucleotides 849 and 2332 of the TEV genome, as well as a larger plasmid containing a full length DNA copy of the TEV-G↓HC genome. These plasmids were grown in *Esherichia coli* strains HB101 and DH5α.

Using the method described by Kunkel [9] variants of the small plasmid containing the desired mutations were made. To create the FRN mutant, the TEV sequence from nucleotides 1600 to 1608, which codes for phenylalanine-arginine-asparagine, was mutated to encode arginine-proline-alanine. The sequence coding for TEV nucleotides 1933-1941, which coded for three cysteines, was similarly mutated to generate the CCC mutation. The arginine-proline-alanine replacements were designed to destroy the function of the replaced sites by disrupting the local primary and secondary structure of the protein. The mutated sequences were then subcloned into the large plasmid, resulting in plasmids that contained mutated copies of the virus.

Confirmation of proper cloning and mutagenisis was performed by digestions with restriction endonucleases and by nucleotide sequencing by the dideoxy method. While sequencing we found that the plasmid with the CCC mutation had an extra mutation 12 nucleotides downstream of the CCC site which changes the codon from glutamate to aspartate. We changed the name of the resulting virus from TEVG↓H-CCC to TEVG↓H-CCC/E to reflect this

change.

Using SP6 RNA polymerase, the full length plasmids containing the mutations were transcribed to produce mutant viral RNAs.

These viral RNAs were used to infect tobacco protoplasts from *Nicotiana tabacum* nc xanthi in order to test the ability of the mutants replicate in single cells. Protoplasts, which are individual plant cells that have been enzymatically stripped of their cell walls, can support viral replication. At timepoints of 24, 48 and 72 hours after innoculation a portion of the protoplasts were harvested, lysed, and frozen. After the last timepoint, the samples were thawed and incubated with the fluorometric substrate 4-MUG (4-methylumbelliferyl beta-D-glucose). The amount of 4-Mug cleaved by the viral GUS protein was recorded with a flourometer. Along with the mutant viruses, protoplasts were inoculated with wild-type TEV-G↓H and TEV-G↓H-VNN, a virus which is defective in replication due to a mutation in the viral replicase gene.

To assay the ability of the mutant viurses to move from cell to cell and through the vascular tissue of the plant, we infected plants with wild type and mutant virus constructs by applying solution containing infectious transcripts onto leaves that had been lightly abraded with carborundum. Cell-to-cell movement was assayed by infiltrating inoculated leaves with GUS substrate which caused infected cells to turn blue. Long distance movement of the viruses was scored by looking for the presence or absence of vein clearing

Table 1: Replication of viruses in protoplasts as measured by GUS assay[1]

Virus	24hr.	48hr.	72hr.
TEVG↓H-VNN	.04	.04	.15
TEVG↓H	2.0	175.0	985.0
TEVG↓H-FRN	5.5	249.0	607.0
TEVG↓H-CCC/E	7.7	225.0	336.0

[1]The GUS assay measures the concentration (pMol/min/100,000 protoplasts) of the cleavage product of the GUS substrate, 4-MUG, using a fluorometer.

in leaves above the infected leaves. In leaves with vein clearing, the veins were noticeably lighter than the surrounding tissue, and produced a mosaic pattern on the infected leaf.

RESULTS

Protoplasts from *N. tabacum* nc xanthi infected with transcripts from the mutant viruses showed levels of replication comparable to those of the wild type TEVG↓H, and levels of replication many times greater than the defective TEVG↓H-VNN at time points of 24, 48, and 72 hours (Table 1).

A single leaf inoculated with TEVG↓H showed two infection foci as determined by a GUS infiltration assay. The leaf inoculated with TEVG↓H-FRN showed no infection foci, while the TEVG↓H-CCC/E inoculated leaf had three infection foci. The presence of macroscopic infection foci indicated that the virus was spreading from the initially infected cell to neighboring cells. It is possible that the lack of infection foci on the FRN inoculated leaf was due to lack of initial infection rather than inability to move from cell to

Table 2: Percentage of inoculated plants showing vein clearing.

Virus	72hr.	111hr.	120hr.
TEVG↓H	0.0	100	100
TEVG↓H-FRN	0.0	0.0	0.0
TEVG↓H-CCC/E	0.0	0.0	0.0

cell.

At 111 hrs. post inoculation all six plants infected with TEV7DAG↓H showed vein clearing in the leaf directly above the inoculated leaf (Table 2). This vein clearing is indicative of systemic spread. None of the plants inoculated with the FRN and CCC/E mutants showed any symptoms of systemic spread. At 118 hours post inoculation, symtoms were absent in the plants infected with the mutants, while infection was progressing in the plants infected with wild type virus.

DISCUSSION

Based on the protoplasts replication assays, neither of the mutant constructs is defective in virus replication at the single cell level. This indicates that neither the FRN site nor the CCC site have any effect on replication, and suggests that HC-Pro (at least the central part of it) does not function as an accesory factor to replication. Our data suggest that both sites are essential for systemic movement of the virus. The mutated CCC site does not appear to hinder cell to cell movement of the virus, but as we only assayed one leaf inoculated with the FRN mutant it is unclear as to whether the cell

to cell movement of the virus is defficient or the leaf escaped infection. We are planning more experiments to determine this.

The function of HC-Pro in systemic movement might explain why the central part of HC type proteins, well conserved among potyviruses, is not conserved in the fungal HAV, since fungi and plants have different transport structures. Along with HC-Pro, the capsid protein is necessary for aphid transmisibility [2], and long distance movement [5]. This leads to the possibility that HC-Pro may interact with the capsid protein to form the complex or complexes needed for aphid transmissibility and long distance transport.

The experiment characterizing the long distance transport of TEV and the HC-Pro mutants is currently in progress.

At present full length plasmids containing mutations at three conserved sites located between the FRN and CCC sites are being constructed along with a construct that has the CCC mutation without the extra glutamate to aspartate substitution. Transcripts of these constructs will be tested in protoplasts as well as in plants for both cell to cell and long distance movement. We hope that these constructs will allow better characterization of the movement function of HC-Pro as well as determine the effect of the gluamate to aspartate replacement.

Bibliography

[1] Allison, R., R.E. Johnston, and W.G. Dougherty. 1986. The Nucleotide Sequence of the Coding Region of Tobacco Etch Virus Genomic RNA: Evidence for the Synthesis of a Single Polyprotein. Virology *154*:9-20.

[2] Atreya, C.D., P.L. Atreya, and T.P. Pirone. 1991. Amino Acid Substitutions in the Coat Protein Result in Loss of Aphid Transmissibility of a Plant Virus. Proc. Natl. Acad. Sci. USA *88*:7887-7891.

[3] Carrington, J.C., and D.D. Freed. 1990. Cap-independent Enhancement of Translation by a Plant Potyvirus 5' Nontranslated Region. J. Virol. **64**:1590-1597.

[4] de Mejia, M.V.G., E. Hiebert, D.E. Purciful, D.W. Thornbury, and T.P. Pirone. 1985. Identification of Potyviral Amorphous Inclusion Protein as a Nonstructural, Virus-specific Protein Related to Helper Component. Virology **142**:34-43.

[5] Dolja, V.V, R. Haldeman, N.L. Robertson and J.C. Carrington 1994. Distinct Functions of Capsid Protein in Assembly and Movement of

Tobacco Etch Potyvirus in Plants. EMBO **13**:1482-1491. EMBO.

[6] Dolja, V.V, and J.C. Carrington. 1992. Evolution of Positive-strand RNA Viruses. Semin. Virol.**3**:315-326.

[7] Dolja, V.V., K.L. Herndon, T.P. Pirone and J.C. Carrington. 1993. Spontaneous Mutagenisis of a Plant Potyvirus Genome after Insertion of a Foreign Gene. J.Virol. **67**:5968-5975.

[8] Koonin, E.V., G.H. Choi, D.L. Nuss, R. Shapira, and J.C. Carrington. 1991. Evidence for Common Ancestry of a Chestnut Blight Hypovirulence-associated Double-stranded RNA and a Group of Positive strand RNA Plant Viruses.

[9] Kunkel, T.A., J.D. Roberts, and R. Zakour. 1987. Rapid and Efficient Site-specific Mutagenesis Without Phenotypic Selection. Methods Enzymol. **154**:367-382.

[10] Lewin, B. 1994. *Genes V* Oxford University Press, New York.

[11] Pirone,T.P., and D.W. Thornbury. 1983. Role of Virion and Helper Component in Regulating Aphid Transmission of Tobacco Etch Virus. Pytopathol. **73**:872-875.

[12] Robaglia, C., M. Durand-Tardif, M. Tronchet, G. Boudazin, S. Aster-Manifacier, and F. Casse-Delbart. 1989. Nucelotide Sequence of Potato Virus Y (N strain) genomic RNA. J. Gen. Virol. **70**:935-947.