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TITLE: Tomato Bushy Stunt Virus and DI RNAs as a Model for Studying Mechanisms of RNA Virus Replication, Pathogenicity and Recombination.

PIs: T.J. Morris, School of Biological Sciences, University of Nebraska, Lincoln, NE. 68588
A.O. Jackson, Dept. of Plant Biology, University of California, Berkeley, CA, 94720.

INTRODUCTION

Tomato bushy stunt virus (TBSV) is small icosahedral virus with a very broad host-range. The symptoms of systemic infection range from mild mosaic to severe necrosis that often results in death. The genome of TBSV is composed of a single plus stranded RNA molecule with five genes. Two 5' genes are translated from the viral RNA, and the remaining three are translated from two subgenomic RNAs. Prior to the DOE supported studies, TBSV gene functions had been assigned solely on the basis of sequence similarity with other virus genes of known function. The two 5' proximal genes (p33 and p92) were thought to be involved in viral replication, the middle gene encoded the capsid protein (p41), but no clear function was assigned to two nested 3' genes (p19 and p22), although it was suggested that at least one could be involved in movement. The DOE supported research has now determined the roles of each of the viral genes in the infection process, and we have obtained considerable genetic information pertinent to the contributions of the coat protein and the nested genes to the disease phenotypes observed in several host plants. We have also identified another genetic element with a short open reading frame in the 3'-noncoding region of the genome that provides a host-dependent replication function..

During passage experiments undertaken several years ago, we noted that attenuation of TBSV occurred upon the generation of defective interfering RNAs (DIs). We were the first to describe and characterize DI RNAs reproducibly generated in association with an RNA plant virus. The features of the DIs that we discovered were compatible with the properties of DIs identified in many animal virus infections. Animal virologists generally recognized the importance of studying DIs because they are invaluable tools for identifying cis-acting sequences important in virus multiplication and because they offer the opportunity to elucidate mechanisms involved in viral persistence and disease attenuation. Hence, our discovery provided a very valuable tool for use in plant virus studies. We have since investigated the mechanisms of origin and evolution of the DIs in addition to the roles these DIs and various mutant derivatives of TBSV have on the disease phenotype. We have also conducted studies to determine the feasibility of using various constructs to protect transgenic plants against the lethal effects of infection.

Our discovery of DIs in plant viruses formed the basis the DOE grant awarded in 1988 with renewal awards in 1991 and 1994. Our previous contributions established plant viral DI RNAs as valuable models for understanding the molecular basis of DI

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mediated interference and offered the potential for developing novel strategies for plant disease control. In the previous renewal, we proposed the following 4 objectives:

Objective 1. Characterization of Structures Important in Replication of DI RNAs

We proposed to identify the *cis*-acting elements in DI RNAs that influenced replication and subsequent accumulation in plants and protoplasts. The analysis of cis-elements important in replication were defined for DI RNAs derived from both CNV and TBSV in the following papers:

Paper #1: Chang et al. did a comparative analysis of deletions targeting the individual conserved regions in both CNV and TBSV DI RNAs. This study revealed that only region III was completely dispensable for accumulation of either DI species. Refined deletion analyses in regions I and II indicated that smaller segments could be deleted without abolishing infectivity. The dispensable sequences in region II of both DI RNAs mapped to the top portion of a putative stem-loop structure. These studies indicated that both essential and non-essential sequences are conserved in DI RNAs. The essential sequences in regions I, II and IV likely contain important cis-acting elements whereas non-essential regions such as region III may play secondary roles such as optimally spacing cis-acting elements or maintaining the DI RNA at a stable size. More recent results suggest that it might also be involved in initiating assembly of the DI RNA into virus.

Paper #11: This study was initiated to further characterize the conserved structural element in region II of the DI RNAs identified by Chang et al. This element has now been further characterized in molecular detail (Robertson, M.S. Thesis) and is being prepared for publication (Robertson, White & Morris, in preparation).

Objective 2: Investigation of the *de novo* Generation and Evolution of DI RNAs

We made substantial progress toward understanding the generation and evolution of DI RNAs. We have applied this knowledge toward exploring basic mechanisms of RNA recombination in the last grant period. We developed a step-wise deletion model to explain the formation of prototypical DIs from larger precursors and showed that DIs could evolve to both smaller and larger forms in both plant and protoplast infections. As part of this objective we proposed to study both recombination between DI RNAs as well as their evolution mediated by DI - viral - host interaction. A brief summary of the 5 papers published during the grant period follows as an overview of the accomplishments.

Paper #2: Law & Morris provided convincing evidence using PCR that DIs could arise de novo in plants inoculated with transcript inocula derived from clones in as early as 8 days post inoculation. These results confirmed that newly formed DIs acquired the same basic specific pattern of sequence modules that we now recognize as being a "prototypical" DI RNA representative of all Tombusviruses. The results also showed that a heterogenous population of DI "quasi-species" accumulates in plants in the absence of serial host passage.

Paper #3: White & Morris used a protoplast system to further analyze the generation and evolution of TBSV DIs. These studies revealed the following: i) that replication competence was the most important factor in DI competitiveness and likely a primary determinant of DI evolution; ii) that DI RNAs are capable of evolving into both larger and smaller forms with different rates; iii) that recombination between DI RNAs was responsible for the evolved forms and iv) that sequence complementarity between plus and minus strands of the DI RNAs might mediate selection of the recombination sites. These results permitted the formulation of a "stepwise deletion model" that explained the temporal order of events leading to DI RNA formation.

Paper #4: White & Morris further analyzed the evolution of atypical DI RNAs that accumulated during passage in protoplasts. We showed in competition assays that segment duplication or single nucleotide insertions could enhance DI competitiveness. Possible mechanisms for the introduction of these modifications were noted. Interestingly, the region most effected included the structure recognized by Chang et al., in paper #1.

Paper #5: White & Morris further developed the protoplast system to analyze mechanisms important in trans-recombination between two distinct Tombusviruses - TBSV and CNV. Our previous studies had not proved that tombusvirus genomes were capable of trans-recombination. We designed experiments in which we co-inoculated protoplasts with *in vitro* generated transcripts of nonreplicating 3'-truncated genomic RNA of CNV and replication defective, 5'-truncated DI RNAs of TBSV. We then identified replicative chimeric recombinants in the protoplasts at 48hr pi. These results showed quite dramatically that: i) RNA recombination could occur efficiently in single cell infections; ii) trans-recombination could occur between non-replicating viral RNA components and iii) functional chimeric genomes between distinct viruses could be generated via recombination. This important study has received some attention in the literature because of the implications for RNA virus evolution and concerns about safety issues involving release of transgenic plants expressing portions of plant viral genomes.

Paper #10. White & Morris continued development of the use of the protoplast system described in paper 4 with a more detailed analysis of sequences and structures important in modulating the frequency of recombination between modified TBSV DI RNAs and defective helper CNV genomic RNA. This study has revealed that both sequence complementarity and RNA structure at the junctions effects the frequency of the recombination events.

Objective 3. Evaluation of TBSV and DI replication and movement

During this phase of the proposed studies, we made significant progress in our understanding of the genetics of TBSV, how the virus causes disease, which factors are responsible for activation of resistance genes, and in the application of viral components for use in pathogen derived resistance strategies. These studies, detailed in **papers 6, 7, 8 & 9,** are summarized below:

Genetic analysis of TBSV replication and movement

Paper # 6. Mutagenesis experiments demonstrated that both 5' genes (p33 and p92) are required for replication of TBSV. Immunodetection using antisera raised against p92 and p33 showed that both proteins are present in the tissue fractions enriched for membrane-associated proteins. We determined that p33 is translated directly from the genomic RNA and that the larger protein (p92) is the result of a translational read-through event that is not very efficient because p33 is present in great excess compared to the levels of p92.

Paper # 13. We also identified an RNA element that regulates replication. A small ORF (pX) near the 3' end of the genome was shown to be required for replication in *Nicotiana sp.* but not in *Chenopodaceae.* Genetic tests revealed that the RNA sequences and not the putative pX protein are responsible for this phenomenon. Additional deletion analyses also identified regions on the genome that interfered with the ability of DI RNAs to be replicated *in trans* by a replication competent TBSV derivative. Elimination of such sequences resulted in the generation of defective RNA molecules that were highly activated upon co-infection with TBSV.

Paper # 7. This paper relates to the requirement of p19 and p22 in movement of TBSV. We constructed a reporter virus in which the CP gene was replaced with the b-glucuronidase (GUS) reporter gene and used histochemical assays to monitor cell-to-cell and vascular movement. Mutagenesis experiments using this construct revealed that p22 is required for this process in all host plants investigated. Deletion of p19 did not have obvious effects on movement in N. benthamiana or N. clevelandii, however, p19 was required for systemic spread in pepper and spinach. We also cloned the p22 and p19 genes into a PVX vector and demonstrated that the p22 could function in trans to complement TBSV mutants unable to express p22. From this work, we concluded that p22 is an absolute requirement for cell to cell movement, whereas the p19 protein has a host-specific role in systemic invasion. We are proposing an several experiments to address the roles of these proteins in more detail in the renewal proposal.

Paper # 8. Immunodetection assays with antisera raised against p19 and p22 showed that p22 is mostly associated with membranes and cell wall components, whereas p19 is a cytoplasmic protein. These results verified our previous conclusions that p22 has characteristics similar to those of most movement proteins. The solubility of the p19 protein suggests that it may interact with proteins in the cytosol. Coinfection withTBSV and the DI's in protoplasts resulted in a dramatically reduced level of sgRNAs encoding the capsid and nested genes and their corresponding encoded proteins. In contrast, a much less dramatic reduction was noted in the replicase (p33 and p92) proteins and in the level of genomic RNA. These results suggested that the protective effects exerted by the DI's may be due in part to selective inhibition of p19 and p22 expression and in part to reduced replication of genomic RNA. The reduced levels of p19 could in turn result in much less extensive necrosis and a higher proportion of surviving plants.

Identification of TBSV genes that elicit host symptom responses.

Paper 9. Infection of *N. benthamiana* and N. clevelandii with TBSV results in a top-necrosis while mutants in which p19 has been deleted only induce a mild systemic mosaic. Our infectivity studies in which p19 and p22 were expressed from the PVX

vector revealed that expression of p19 changed the normal mild mosaic response of PVX to a systemic tissue collapse followed by host cell death that resembled the symptoms induced by TBSV. The combination of genetic studies on the TBSV genome and the PVX mediated expression demonstrated that p19 is responsible for the onset of the systemic necrotic lesion response in *N. tabacum*. However, in two other hosts that respond with local lesions upon inoculation with TBSV, *N. glutinosa and N. edwardsonii*, p22 rather than p19 elicited the hypersensitive response. These results thus suggest that closely related plant species contain different resistance genes that effectively combat TBSV infection.

Work in Progress:

Recombination of defective tombusviruses with a host transgene.

Paper 14. During the project period, we conducted a series of experiments to evaluate the expression of the capsid protein in N. benthamiana. Interestingly, of more than 70 transgenic lines expressing mRNA, none showed symptoms related to expression of the transgene, and we were unable to detect coat protein expression in selected plants. In addition, none of the plants exhibited substantial resistance to wild type TBSV infection, and all inoculated plants died within ten days to two weeks after inoculation. However, following inoculation with TBSV HS7, a mutant with a frame shift that abrogated expression of the capsid protein gene, mild symptoms were observed that resembled those routinely detected in nontransgenic plants. Similar results were obtained with a CNV chimeric derivative containing a TBSV coat protein gene with the same frame shift mutation. In both cases however, up to 15% of the inoculated plants began to exhibit a necrosis which appeared sporadically beginning about two weeks after inoculation. These plants all died and all were characterized by the presence of high concentrations of wild type virus. Upon examination using a variety of different procedures, we concluded that the defective viruses had undergone recombination with the coat protein transgene to restore expression of the coat protein cistron. Several tests using a CAT derivative and the CNV derivative verified these conclusions. A manuscript describing these experiments has been submitted to MPMI and is being revised for publication. We intend to revise the present results and plan to include an additional experiment suggested by one of the reviewers to determine whether heterologous recombination occurs between the host transgene and a CNV mutant containing a CNV capsid protein with a frame shift similar to that of TBSV. The editor of MPMI has also invited us to discuss the significance of our findings in the Perspectives of the issue in which the paper appears.

Pathogen derived resistance

Paper 15. The ability of TBSV DIs to protect transgenic plants from infection was investigated to determine the feasibility of using cloned cDNAs of naturally occurring defective interfering (DI) RNAs of TBSV to engineer resistance to TBSV infection in transgenic plants. The strategy we chose to employ uses ribozymes from satellite tobacco ringspot virus and avocado sunblotch viroid to flank the DI transcript at the 3' and 5' termini to produce biologically active DI's that exactly represent the wild B10 DI used for construction of the transgenic plants. We have carried out a large number of

experiments which demonstrate that these plants have a high level of resistance to the lethal necrosis caused by wild type TBSV. The plants initially show a characteristic severe mosaic, but in contrast to nontransgenic plants, the DI containing derivatives recover and high levels of amplified DI RNAs are present in the recovering leaf tissue. Moreover, a broad spectrum resistance to at least five Tombusviruses has now been obtained in transgenic plants expressing DI RNA based constructs. This aspect of our research thus may be suitable for commercial use in vegetables and fruits that suffer from diseases caused by TBSV. Individuals conducting these and most of the molecular genetics experiments in the AOJ lab were supported in part by an NSF CEPRAP project designed to engineer plants with resistance against plant pathogens.

Objective 4. Application of yeast as a genetic tool for analysis of TBSV replication.

For this approach we spent considerable time testing the feasibility of yeast as a tool for genetic analysis of TBSV because the powerful genetic approach could have been very useful for identification of host genes required for replication of the virus. After more than a year of intensive research, we have reluctantly come to the conclusion that this system is not useful for this purpose with TBSV. The basic problem seems to be that the reporter gene (URA3) used as a selectable marker for TBSV replication is expressed in limited but sufficient amounts to permit yeast growth even from control transcripts with deletions in the replicase gene. Thus, we are abandoning this approach and will be describing our results at the American Society for Virology Meetings.

Publications for the Grant Period

1. Chang, Y.-C., Borja, M., Scholthof, H.B., Morris, T.J., and Jackson, A.O. (1995). Host effects and sequences essential for accumulation of defective interfering RNAs of cucumber necrosis and tomato bushy stunt tombusviruses. *Virology* 210: 41-53.

2. Law, M.D. & Morris, T.J. (1994). De novo generation and accumulation of tomato bushy stunt virus defective interfering RNAs without serial passage. Virology 198: 377-380.

3. White, K.A., & Morris, T.J. (1994). Nonhomologous RNA recombination in Tombusviruses: Generation and evolution of defective interfering RNAs by step-wise deletions. J. Virol. 68: 14-24.

4. White, K.A., & Morris, T.J. (1994). Enhanced competitiveness of tomato bushy stunt defective interfering RNAs by segment duplication or nucleotide insertion. J. Virol. 68: 6092-6096.

5. White, K.A., & Morris, T.J. (1994). Recombination between defective Tombusvirus RNAs generates functional hybrid genomes. PNAS 91: 3642-3646.

6. Scholthof, K.-B. G., Scholthof, H.B., and Jackson, A.O. (1995). The tomato bushy stunt virus replicase proteins are coordinately expressed and membrane associated. Virology 208:365-369.

7. Scholthof, H. B., Scholthof K-B. G., Kikkert, M., and Jackson, A. O. (1995). Tomato bushy stunt virus spread is regulated by two nested genes that function in cell-to-cell movement and host-dependent systemic invasion. *Virology* **213**: 425-438.

8. Scholthof, K.-B. G., Scholthof, H.B., and Jackson, A.O. (1995). The effect of defective interfering RNAs on the accumulation of tomato bushy stunt virus proteins and implication for disease attenuation. Virology 211:324-328

9. Scholthof, H.B., Scholthof K.-B. G., and Jackson, A.O. (1995). Identification of tomato bushy stunt virus host-specific symptom determinants by expression of individual genes from a potato virus X *vector. Plant Cell* **7**: 1173-1184.

10. White, K.A., & Morris, T.J. (1995). RNA determinants of junction site selection in RNA virus recombinants and defective interfering RNAs. RNA 1: 1029-1040.

11. Robinson, F.L.(1996). Essential sequences in region II of a defective interfering RNA of tomato bushy stunt virus. (MS Thesis 1995, manuscript in preparation, abstract attached).

12. Scholthof, H. B., Scholthof, K-B G., and Jackson, A. O. (1996). Plant virus gene vectors for transient expression of foreign proteins in plants. *Ann Rev. Phytopathology* **34**: 299-323.

13. Scholthof, H. B., and Jackson, A. O. (1997). The enigma of pX: A host-dependent cis-acting element with variable effects on Tombusvirus RNA accumulation. Virology (Accepted Pending Revision).

14. Borja, M., Rubio, T. Scholthof, H. B., and Jackson . A. O. (1997). Recombination of a host coat protein transgene with a coat protein deficient virus to generate wild type virus. MPMI. (Accepted Pending Revision)

15. Rubio, T., Borja, M., Scholthof, H. B., Feldstein, P., Bruening, G., Morris, T.J. and Jackson . A. O. Broad spectrum resistance to tombusviruses in defective interfering RNAs transgenic plants. (Manuscript submitted).

Numerous additional abstracts were presented at meetings. We have not listed them but can provide such on request.