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Molecular Approaches for Control of Tomato Bushy Stunt Virus

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CHAPTER 23

Molecular Approaches for Control of Tomato Bushy Stunt Virus

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23.1 INTRODUCTION

In this overview strategies are discussed that are currently being tested in our laboratory in an effort to engineer resistance against tomato bushy stunt virus (TBSV) in plants. TBSV is chosen as a model-system since this is an economically important RNA virus that is well characterized at the molecular level and the virus has some interesting and potentially useful features that can be exploited to produce resistant plants. Moreover, we anticipate that the information derived from our studies with TBSV will be applicable in developing resistance to other plant viruses.

Some approaches that we are attempting to apply to TBSV have been described previously for other virus-systems, whereas other strategies represent novel tactics. This overview will also illustrate that some methods are in an advanced stage of implementation, whereas others are still being tested at a very elementary model-system level. The effectiveness of most strategies remains to be evaluated but we will present our progress to date, consider the implications of certain findings for future developments, and point out directions we intend to take with the TBSV program.

23.2 TOMATO BUSHY STUNT VIRUS

23.2.1 GENE FUNCTIONS

Tomato bushy stunt virus (TBSV) is a pathogen of tomatoes which is associated with the appearance of tomato decline disease in the field where

it causes severe stunting and yield losses (Gerik et al., 1991). TBSV has a broad host range (reviewed by Martelli et al., 1988) and in the hosts *Nicotiana clevelandii* and *N. benthamiana*, TBSV incites a lethal necrosis which kills the plants generally within two weeks after inoculation. TBSV, the type member of the tombusvirus group, is a small isometric virus and, as shown in Figure 23.1, it contains a single positive sense RNA genome of ca. 4800 nucleotides encoding five genes (Hearne et al., 1990). A full-length cDNA clone of TBSV is available and infectious RNA transcripts are routinely used for infectivity assays in plants and protoplasts. Recently we have developed a tomato protoplast system which allows us to study viral functions and replication in these host cells (H. Scholthof, unpublished data).

The larger of the two 5′ proximal TBSV gene products (p92) (Figure 23.1) results from readthrough of a termination codon. Replication studies have demonstrated that both 5′ genes (p33 and p92) are required for replication (Scholthof et al., 1991). The third gene encodes the coat protein gene (p41) which is translated from a subgenomic mRNA (Figure 23.1) and it is dispensable for replication and systemic movement (Scholthof et al., 1992 and manuscript in preparation). The 3′ terminal region encodes a 19 kD gene product whose open reading frame (ORF) is out of phase but completely nested within that of a larger 22 kD gene product (Figure 23.1). Consequently, both 3′ terminal genes are translated from the same subgenomic mRNA, and at least one of these appears to be required for systemic movement (Scholthof et al., manuscript in preparation) as also suggested for cucumber necrosis tombusvirus (Rochon and Johnston, 1991).

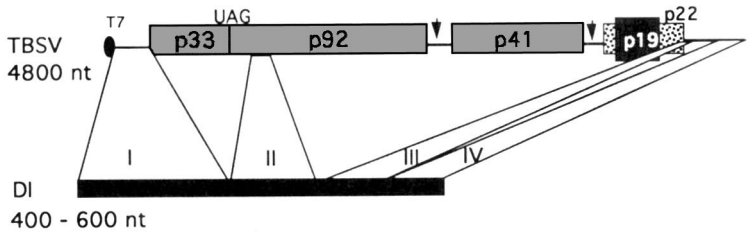

Figure 23.1. Schematic representation of the tomato bushy stunt virus (TBSV) genome and associated defective interfering RNAs (DIs). The diagram at the top depicts the ca. 4800 nucleotide (nt) genome of TBSV. Boxes indicate the open reading frames (ORFs). The two 5′ proximal ORFs, p33 and p92, are involved in replication (UAG is the amber codon) and p41 gene encodes the coat protein. At least one of the overlapping 3′ end genes (p19 and p22) is involved in systemic movement. Arrows denote the approximate position of subgenomic promoters. The position of the T7 promoter on the infectious cDNA plasmid is indicated, vector sequences are omitted from the diagram. The highlighted areas represent the conserved genomic blocks (I through IV) which constitute the DIs represented on the bottom.

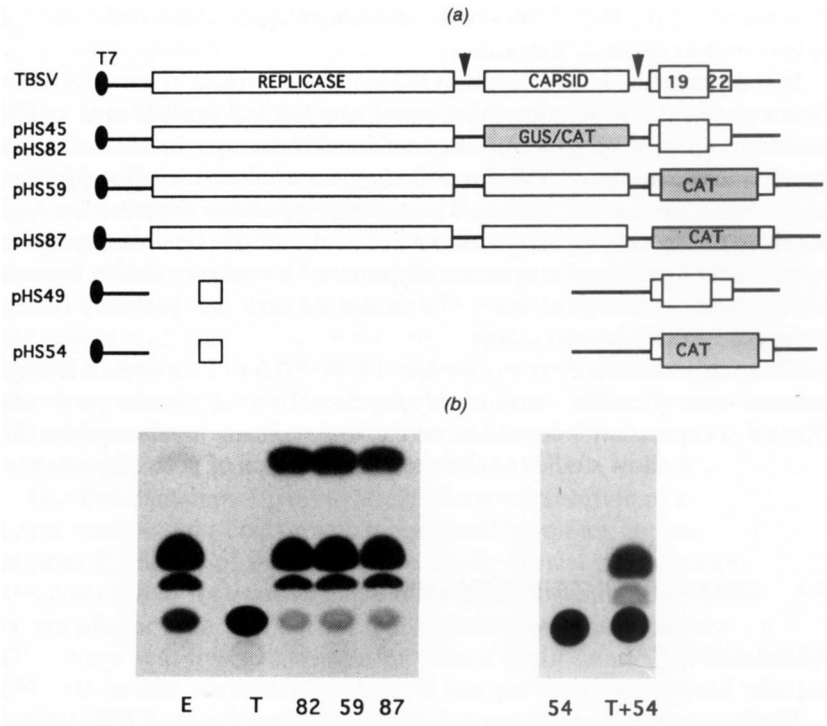

Figure 23.2. Expression of foreign genes from TBSV and from defective RNAs. (a) Diagram of the constructs used in the gene expression studies, symbols are as in Figure 23.1. In pHS45 the coat protein is replaced with the β-glucuronidase (GUS) gene (data not shown) and in pHS82 it is replaced with the gene for chloramphenicol acetyltransferase (CAT). In pHS59 and pHS54 the CAT gene is positioned in-frame with the AUG of p19 whereas pHS87 contains the reporter gene in frame with p22. Open spaces indicate genomic sequences that are deleted in pHS49 and pHS54. (b) CAT assay of extracts from protoplast transfected with transcripts from the following plasmids: pTBSV, pHS82, pHS87, pHS59 (all 10 μg); pHS54 (25 μg); PTBSV (10 μg) plus pHS54 (25 μg); E, 0.5 μg purified CAT enzyme.

23.2.2 EXPRESSION OF FOREIGN GENES BY TBSV

To examine the gene vector potential of TBSV, we replaced either the coat protein gene or the 3′ nested ORFs with a reporter gene as shown in Figure 23.2. In protoplasts all these constructs replicated efficiently and they also gave rise to a high level of reporter gene expression (Figure 23.2) that was dependent on an intact replicase gene (Scholthof et al., 1991 and manuscript in preparation). Replacement of the 3′ ORFs with a reporter gene resulted in viral RNAs that were unable to induce systemic symptoms on *N. benthamiana* or local lesions on *Chenopodium amaranticolor*. This confirms

the observation that at least one of these overlapping ORFs of tombusviruses is involved in systemic movement.

In contrast to the 3′ overlapping ORFs, the coat protein of tombusviruses is not required for infection (McLean et al., 1992; Scholthof et al., 1991 and manuscript in preparation). Recombinant transcripts in which the coat protein gene is replaced with a reporter gene are able to incite local lesions as well as a systemic infection. The reporter genes are expressed to high levels in the inoculated leaves (Scholthof et al., 1992), but this expression is dramatically reduced in systemically infected leaves presumably because of the rapid *in vivo* generation of deletion mutants that probably have a competitive selective advantage.

These experiments demonstrate that TBSV RNA can replicate a foreign gene whose expression is presumably regulated by a subgenomic promoter. Since this expression is dependent on the viral replicase it indicates that this system might allow studies on the specific activation of gene expression.

23.3 DEFECTIVE INTERFERING RNAS

23.3.1 GENERAL

During routine maintenance on plants in the greenhouse, TBSV isolates appear that cause attenuated symptoms which culminate in a persistent infection rather than the normal lethal necrosis (Hillman et al., 1987). These attenuating isolates contain massive amounts of defective interfering RNAs (DIs), derived from the parental genome (Figure 23.1), which are the actual cause of the interference with the disease symptoms (Hillman et al., 1987; Morris and Hillman, 1989).

Infectious cDNA clones of several DI isolates have been generated and co-inoculation of TBSV RNA with DI transcripts from these clones results in the same dramatic attenuation of symptoms as with naturally occurring DIs (Morris and Knorr, 1990). DIs have also been observed for other members of the tombusvirus group (Rochon and Johnston, 1991; Burgyan et al., 1989) and their generation appears to be a generally occurring phenomenon for this group of viruses. TBSV DIs vary in size from ca. 400 to 600 nucleotides and consist of conserved blocks of genomic sequences (I through IV in Figure 23.1) that do not code for any proteins (Knorr et al., 1991). The DIs depend on the helper virus for replication and they very efficiently interfere with replication of TBSV in protoplasts (Jones et al., 1990). This impediment of viral RNA replication is believed to be responsible for the interference of DIs with symptom induction in plants.

23.3.2 TBSV MEDIATED GENE EXPRESSION FROM DEFECTIVE RNAS

For several purposes we are attempting to use DI RNAs, or synthetic replicase defective viral RNAs, to express foreign genes in presence of wild-type virus. One of our goals is to generate transgenic plants that express a defective RNA containing a reporter gene under control of a subgenomic promoter. This gene will not be expressed until the virus infects the cell which will lead to replication and gene expression of the foreign gene. This type of gene activation will allow a detailed study of virus movement without debilitating the virus itself. In the previous sections we have already shown that TBSV can express and replicate a foreign gene and the virus replicase is active in *trans* for replication of DIs. The next question is whether foreign gene expression can be activated from defective RNAs whose replication is dependent on the virus.

Our initial attempts involved straightforward insertion of a foreign gene under control of the coat protein subgenomic promoter, between conserved regions II and III of the DI (Figure 23.1). However, transcripts of this construct fail to replicate or express the foreign gene in protoplasts when co-transfected with TBSV RNA. Subsequently we have established that the DI is very sensitive to insertion of even a small number of nucleotides (40−50) at that particular location. It has yet to be determined whether insertions at different positions will be less detrimental.

During our studies on factors involved in replication (Scholthof et al., manuscript in preparation), we have generated a synthetic defective RNA resembling a naturally occurring DI but which still contains the subgenomic promoter and complete coding region for the 3′ ORFs (plasmid pHS49 in Figure 23.2). Transcripts of this construct replicate to very high levels in presence of the virus (data not shown). This plasmid is an attractive candidate for our purposes since it might be able to express a foreign gene from its subgenomic promoter. To test this possibility plasmid pHS54 (Figure 23.2) is generated which has the gene for chloramphenicol acetyltransferase (CAT) in place of the 3′ ORFs. Transcripts of this construct do not replicate by themselves, but in the presence of TBSV they are amplified to extremely high levels exceeding the amount of helper virus RNA. This construct serves as an excellent gene vector whose multiplication is completely dependent on the wild-type virus.

The protoplasts were also assayed for CAT activity. The results in Figure 23.2 demonstrate that there is no CAT expression from pHS54 RNA in absence of TBSV but high levels of expression are obtained in presence of the virus. We are optimistic that derivatives of pHS54 can be used to generate transgenic plants (see also Section 23.4.3.2) whose defective transcripts are being activated by TBSV. This will enable studies on move-

ment of wild-type TBSV when for instance the β-glucuronidase (GUS) gene is used as a reporter gene. Its expression can be easily monitored on the cellular level as well as the whole plant level employing simple GUS dependent colorimetric reactions.

23.4 STRATEGIES AND PROGRESS IN RESISTANCE DEVELOPMENT

23.4.1 COAT PROTEIN MEDIATED RESISTANCE

Cross protection is a phenomenon whereby a mild strain of a virus can protect a plant against subsequent invasion by a more virulent isolate. A few years ago it was demonstrated that cross protection against tobacco mosaic virus (TMV) could also be achieved in transgenic plants expressing the coat protein of the virus (Powell Abel et al., 1986). Since this first observation, numerous other examples of coat protein mediated resistance, involving at least nine different groups of plant viruses, have appeared in the literature (Beachy et al., 1990).

As part of our resistance development program, we are testing whether coat protein mediated cross-protection is applicable for TBSV. We have recently PCR amplified and cloned the coat protein gene of TBSV and verified by *in vitro* translation of corresponding transcripts that this gene encodes the authentic protein (unpublished data). The DNA has been inserted into a vector suitable for *Agrobacterium* mediated transformation of plants. Transgenic plants have been regenerated and these will be subjected shortly to extensive tests to determine if there is a protective effect associated with the expression of the coat protein.

The mechanism of coat protein mediated protection is still unclear and appears to act at different stages during the life-cycle for different viruses (Beachy et al., 1990). The infectious clones of TBSV in conjunction with a reliable protoplast system offer an excellent opportunity to examine the molecular mechanism involved in the putative protection.

23.4.2 DEFECTIVE REPLICASE MEDIATED PROTECTION

A recent report on transgenic plants expressing a portion of the TMV replicase has demonstrated that this approach can give rise to very high levels of resistance against infection with TMV (Golemboski et al., 1990). Although the mechanism behind this type of protection is unclear, it has since proven successful for other plant virus systems (Braun and Hemenway, 1992; MacFarlane and Davies, 1992; Zaitlin et al., 1992). Because the protection is very specific for the virus from which the replicase gene is derived, it is unlikely that this approach will provide a broad resistance phenotype. Nonetheless, this type of approach is a very important addition

to resistance development programs. Moreover, the mechanism responsible for this resistance will undoubtedly be of great value for the development of future engineered resistance strategies.

We are planning to generate transgenic plants that express a portion of the TBSV replicase gene (p92, Figure 23.1). A segment will be chosen whose product will no longer be capable of serving as a replicase enzyme but maintain the capacity to specifically bind to viral RNA. Hopefully the expression of TBSV defective replicases in transgenic plants can deter the virus from establishing a successful infection in a similar manner as for TMV (Golemboski et al. 1990).

As with the previous strategy we will be able to study the degree of resistance and hopefully elucidate the underlying mechanism through studies in transgenic plants as well as in transgenic protoplasts. The specificity can be addressed simply by determining the effect of the transgenic replicase segment on replication of other TBSV strains (Gerik et al., 1991) and other tombusviruses (Martelli et al., 1988).

23.4.3 DI MEDIATED INTERFERENCE WITH REPLICATION

23.4.3.1 Transgenic Plants Expressing DIs

The expression of DIs in transgenic plants offers attractive prospects for virus control since these molecules rapidly accumulate once the virus enters the cell. Therefore, they can interfere immediately during the initial replication cycle. To test the ability of synthetic DIs to control virus replication we have generated transgenic tomatoes which continuously express DI transcripts. However, studies on the whole plant level as well as in protoplasts have demonstrated that these DIs do not replicate in presence of TBSV and consequently we have been unable to detect any protective effect (H. Scholthof, unpublished data).

The lack of replicative ability is probably due to the presence of about 200 extra nucleotides distributed at both termini of the DI RNA. These additional sequences are a result of the engineering steps in the construction of the DNA vectors that have been used for the transformation. The endogenous DI transcripts do not cause any detrimental effect on appearance and fitness of the plants, fruits and seeds. The transgenic plants (R0 and R1) are phenotypically indistinguishable from the normal plants and the yield of fruit and germination of seeds are also equivalent (H. Scholthof, unpublished data).

23.4.3.2 Use of Ribozymes to Recreate Highly Competitive DIs

To overcome the problem of extraneous sequences at both termini of the DIs discussed in the previous section, we have very recently refined our

strategy by generating new DI cDNA constructs which contain *cis*-acting ribozyme sequences on either end, as schematically represented in Figure 23.3. These catalytic entities should cleave the transcripts *in vivo* at the appropriate positions and generate transcripts that possess termini identical to those of native DIs. This processing step will very likely create highly competitive DIs and facilitate their replication to high levels in plants upon inoculation with TBSV. This should enable these molecules to exert their interference with the replication of the helper virus which should dramatically attenuate the onset of disease symptoms.

Preliminary results have shown that DI transcripts containing the ribozyme sequences are processed *in vitro* to generate authentic length DI RNA (unpublished data). Co-inoculation of these molecules with TBSV into protoplasts results in readily detectable levels of amplified DIs as shown in Figure 23.3. Apparently the presence of the ribozyme at the 5′ end was especially important for the replicative ability of these synthetic DIs. In addition, initial infectivity studies indicate that the co-inoculation of TBSV with ribozyme containing DI transcripts onto plants dramatically interferes with symptom induction by the virus. This strongly suggests that these molecules are processed in the plant into biologically active DIs. Currently we are transferring these ribozyme containing DI cDNAs to plant expression vectors. These plasmids will then be transfected into protoplasts which will be assayed for replication of DIs upon co-transfection with viral RNA. Subsequently, the DNA will be used to transform plants to test the level of protection on the whole plant level.

23.4.4 Antisense RNAs

Several examples are documented in the literature where complementary (or antisense) RNA to several plant viruses has been expressed in transgenic plants. It is assumed that this antisense RNA can anneal to the viral genome of the virus which then interferes with essential processes such as translation and replication. The use of antisense RNAs has led to some degree of protection in some instances but not in others (reviewed by Beachy et al., 1990). As part of all the strategies discussed in the previous sections, plants have been or will be transformed with constructs that have the particular viral gene or segment of TBSV in the reverse orientation to test the effect of antisense RNA on an infection with TBSV.

Preliminary tests indicate that expression of antisense DIs (complementary to the DIs of Section 23.4.3.1) in transgenic tomatoes does not significantly affect replication of TBSV in these plants. Similarly, protoplasts of these plants support replication of TBSV and co-transfected (plus sense) DI transcripts in the same manner as normal protoplasts. More extensive evaluations are required but it appears that antisense DIs do not have a

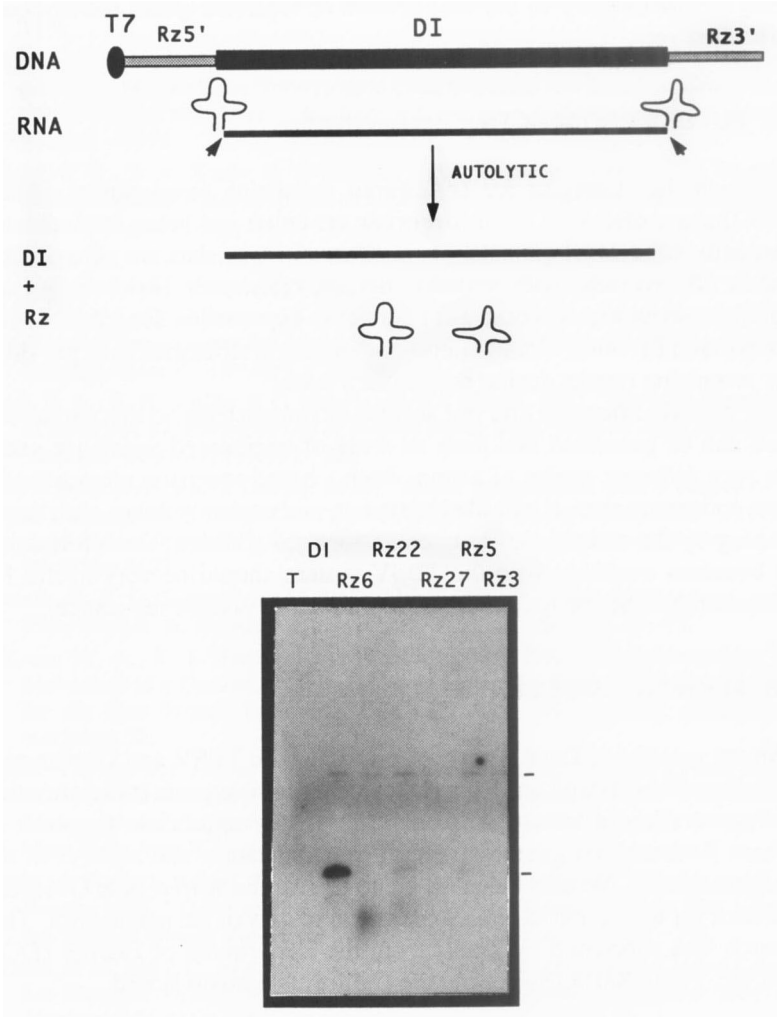

Figure 23.3. Employment of ribozymes to create authentic termini on engineereed DIs. (a) The top bar represents a cDNA clone of a DI with the T7 promoter and the ribozyme sequences (Rz5′ and Rz3′) on either end. The RNA transcript from this DNA is auto-catalytically cleaved (middle bar) to yield authentic DIs (bottom bar) and the ribozyme molecules (cloverleaf structures). (b) Northern analyses of protoplasts that were transfected with *in vitro* generated transcripts of TBSV, co-transfected with different DI transcripts. DI, biologically active DI transcripts that have the correct ends; Rz6, Rz22 and Rz27 are transcripts from constructs with an organization as diagrammed in (a); Rz5, this RNA has the ribozyme at the 5′ end and a native DI 3′ end; Rz3, the ribozyme is present at the 3′ end but extra non-viral sequences are added to the 5′ end. In all cases 10 μg TBSV was used as inoculum together with 25 μg of the respective DI RNA. The position of TBSV genomic RNA and the DIs is indicated by the top and lower bars, respectively.

protective effect against an infection with TBSV. The effect of antisense RNAs complementary to the coat protein or replicase genes has not been established yet.

23.5 FUTURE PROSPECTS

Most of the strategies for engineered resistance development against TBSV that are discussed in this overview are either just being implemented or are still in the developmental phase. Therefore, no data are yet available on their effectiveness with regard to disease resistance. However, we are optimistic about the outcome because some approaches described herein have worked for other virus-systems and the novel strategies have provided very promising results during preliminary tests.

Our goal is to develop and test several model-systems so that eventually plants can be generated that have an array of engineered resistance genes with very different modes of action. Such a broad spectrum approach will be advantageous since it will likely prevent, and certainly delay, resistance-breaking by the virus under field conditions. In addition, the information that becomes available with the TBSV system should be very useful for application in other programs for development of disease resistance.

23.6 ACKNOWLEDGEMENTS

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23.7 REFERENCES

Beachy, R. N., S. Loesch-Fries and N. E. Tumer. 1990. "Coat Protein-Mediated Resistance against Virus Infection," *Annual Review of Phytopathology*, 28:451–474.

Braun, C. J. and C. Hemenway. 1992. "Expression of Amino-Terminal Portions or Full-Length Viral Replicase Genes in Transgenic Plants Confers Resistance to Potato Virus X Infection," *Plant Cell*, 4:735–744.

Burgyan, J., F. Grieco and M. Russo. 1989. "A Defective Interfering RNA Molecule in Cymbidium Ringspot Virus Infections," *Journal of General Virology*, 70:235–239.

Gerik, J. S., J. E. Duffus, R. Perry, D. C. Stenger and A. F. Van Maren. 1990. "Etiology of Tomato Plant Decline in the California Desert," *Phytopathology*, 80:1352–1356.

Golemboski, D. B., G. P. Lomonossoff and M. Zaitlin. 1990. "Plants Transformed with a Tobacco Mosaic Virus Nonstructural Gene Sequence Are Resistant to the Virus," *Proceedings of the National Academy of Sciences of the United States of America*, 87:6311–6315.

Hearne, P. Q., D. A. Knorr, B. I. Hillman and T. J. Morris. 1990. "The Complete Genome Structure and Synthesis of Infectious RNA from Clones of Tomato Bushy Stunt Virus," *Virology*, 177:141–151.

Hillman, B. I., J. C. Carrington and T. J. Morris. 1987. "A Defective Interfering RNA That Contains a Mosaic of a Plant Virus Genome," *Cell*, 51:427–433.

Jones, R. W., A. O. Jackson and T. J. Morris. 1990. "Defective-Interfering RNAs and Elevated Temperatures Inhibit Replication of Tomato Bushy Stunt Virus in Inoculated Protoplasts," *Virology*, 176:539–545.

Knorr, D. A., R. H. Mullin, P. Q. Hearne and T. J. Morris. 1991. "*De novo* Generation of Defective Interfering RNAs of Tomato Bushy Stunt Virus by High Multiplicity Passage," *Virology*, 181:193–202.

MacFarlane, S. A., and J. Davies. 1992. "Plants Transformed with a Region of the 201-Kilodalton Replicase Gene from Pea Early Browning Virus RNA1 Are Resistant to Virus Infection," *Proceedings of the National Academy of Sciences USA*, 89:5829–5833.

Martelli, G. P., D. Gallitelli and M. Russo. 1988. "Tombusviruses," Chapter 4, in *The Plant Viruses*, R. Koenig, ed., New York: Plenum Press, pp. 13–72.

McLean, M. A., R. I. Hamilton and D. M. Rochon. 1992. "Symptomatology and Movement of a Cucumber Necrosis Virus Mutant Lacking the Coding Sequence for the Coat Protein Protruding Domain," ASV 1992 meeting, presentation, workshop 52.

Morris, T. J. and B. I. Hillman. 1989. "Defective Interfering RNAs of a Plant Virus," in *Molecular Biology of Plant-Pathogen Interactions, UCLA Symposia on Molecular and Cellular Biology,* B. Staskawicz, P. Ahlquist and O. Yoder, eds., New York: Alan R. Liss, pp. 185–197.

Powell, A. P., R. S. Nelson, B. De, N. Hoffman, S. G. Rogers, R. T. Fraley and R. N. Beachy. 1986. "Delay of Disease Development in Transgenic Plants That Express the Tobacco Mosaic Virus Coat Protein Gene," *Science*, 232:738–743.

Rochon, D. M. and J. C. Johnston. 1991. "Infectious Transcripts from Cloned Cucumber Necrosis Virus cDNA: Evidence for a Bifunctional Subgenomic mRNA," *Virology*, 181:656–665.

Scholthof, H. B., Y. C. Chang, I. T. D. Petty, P. Q. Hearne, D. A. Knorr, T. J. Morris and A. O. Jackson. 1991. "Comparison of Requirements for Replication and Movement of Genomic versus Defective Interfering RNAs of Tomato Bushy Stunt Virus," *Phytopathology*, 81:1175.

Scholthof, H. B., T. J. Morris and A. O. Jackson. 1992. "Expression of Foreign Genes by Tomato Bushy Stunt Virus and Associated Defective RNAs," *Phytopathology*, abstract (in press).

Zaitlin, M., J. P. Anderson and P. Palukaitis. 1992. "Resistance to Cucumber Mosaic Virus Is Induced in Transgenic Tobacco Plants by a Defective Replicase Gene," ASV 1992 meeting, presentation, workshop 60.