MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 2, No. 4, pp. 209-213, 1989

Point Mutations in the Coat Protein Gene of Tobacco Mosaic Virus Induce Hypersensitivity in *Nicotiana sylvestris*

James N. Culver and William O. Dawson

Department of Plant Pathology, University of California, Riverside 92521 U.S.A.
Received 6 March 1989. Accepted 5 April 1989.

Four point mutations in different regions of the coat protein gene of tobacco mosaic virus (TMV) were created by site-directed mutagenesis. Mutations were made at nucleotide 5745 (guanine to adenine), 5773 (cytosine to uracil), 5788 (adenine to guanine), and 5850 (adenine to guanine) to alter single amino acids in the coat protein at position 11 (Val to Met), 20 (Pro to Leu), 25 (Asn to Ser), and 46 (Arg to Gly), respectively. Infectious transcripts of the four mutagenized cDNA clones produced phenotypically distinct hypersensitive reactions on *Nicotiana sylvestris* (N′ genotype) and systemic responses on *N. tabacum* 'Xanthi' (n genotype). The parental wild-type virus produced systemic mosaic symptoms on both hosts. Sequence data from virion RNA as well as maintenance of phenotype showed that the introduced mutations were stable. This work demonstrates that a number of sites in the coat protein gene of TMV affect host response.

The N′ gene of *Nicotiana sylvestris* Speg. & Comes confers resistance to some strains of tobacco mosaic virus (TMV) but not others, including the common U1 strain. N′ gene resistance is expressed by the appearance of necrotic lesions on leaves that have been inoculated with the appropriate virus. The production of these lesions, known as the hypersensitive reaction (HR), occurs at the sites of viral infections and results in confinement of the virus to these areas. For this reason, incorporation of the HR resistance trait into plants has been a useful means of controlling virus diseases. However, for many virus-host systems, the HR resistance trait is either unavailable or is difficult to transfer into desirable crop species using existing plant breeding techniques (Russell 1978). Because the gene(s) controlling this resistance mechanism could be mobilized via genetic engineering strategies, it is important to understand the mechanisms involved in HR induction.

Current understanding of the mechanisms of HR is mainly limited to the biochemical processes involved in cellular necrosis (Legrand *et al.* 1976). Little is known about the processes involved in HR induction. However, Flor (1971) demonstrated that HR disease resistance occurs via a specific gene-for-gene recognition between host and pathogen. Work done in both fungal and bacterial systems has shown the presence of specific pathogen avirulence genes responsible for determining race-specific HR (Keen and Staskawicz 1988). For these systems, HR disease resistance is obtained when dominant pathogen avirulence genes interact with dominant host resistance genes (Flor 1971). Unfortunately, plant viral systems have not been as well defined. Earlier work with mutant viruses having amino acid substitutions in their coat proteins indicated a possible correlation between these substitutions and the induction of HR (Funatsu and Fraenkel-Conrat 1964).

Recently, the complete cloning of the TMV genome and the ability to produce infectious RNA transcripts from these cDNA clones have created a unique system in which to study virus-induced HR (Dawson *et al.* 1986; Meshi *et al.* 1986). Work done on this level has mapped the induction of HR in *N. sylvestris* to the coat protein gene of TMV (Saito *et al.* 1987). This was demonstrated through the production of coat protein hybrids of TMV-L, which produces HR in *N. sylvestris*, and TMV-OM, which moves systemically in *N. sylvestris*. When the coat protein gene of TMV-L is replaced by the coat protein gene of TMV-OM, HR is not induced.

More specifically, the cloning of nitrous acid mutants of TMV capable of inducing HR in *N. sylvestris* and the subsequent replacement and sequencing of specific segments of these mutant genomes into the original non-HR-inducing cDNA clone of TMV reveal that a single point mutation in the coat protein gene is responsible for the induction of HR (Knorr and Dawson 1988). The identified point mutation resulted in a single amino acid substitution in the coat protein. By coincidence this amino acid substitution was identical to one of several substitutions previously described (Funatsu and Fraenkel-Conrat 1964). Thus, other point mutations in the coat protein gene of TMV might also lead to the induction of HR in *N. sylvestris*. The discovery of other HR-inducing point mutations should provide a first step toward identifying possible molecular and structural requirements for the induction of HR.

This paper describes the results from the production of specific mutations in the coat protein gene of TMV. Mutations were created by the use of site-directed mutagenesis to specifically change selected nucleotides. Four independent nucleotide changes were selected so as to alter the amino acid sequence at four different places throughout the coat protein. These amino acid substitutions were based on previously described coat protein mutants (Funatsu and Fraenkel-Conrat 1964). Each of the four mutations was found to induce HR in *N. sylvestris*.

MATERIALS AND METHODS

Virus strains and plasmids. pTMV204, a full-length infectious clone of TMV U1, was used as the parental clone for the mutagenesis procedure (Dawson *et al.* 1986). A subclone, pDL3, containing the 3′ end of TMV, including the coat protein gene, was constructed by inserting a *Hin*dIII (5,081 bp) to *Pst*I (6,406 bp) fragment from pTMV204 into the polylinker region of pUC119 (Vieira and Messing 1987).

Site-directed mutagenesis and mutation screening. *In vitro* mutagenesis was accomplished via a modified M13 method previously described (Geisselsoder *et al.* 1987). Second-strand synthesis was accomplished using synthetically created oligomers ranging from 16 to 18 nucleotides in length. Each oligomer contained a single mismatched base from the wild-type coat protein gene sequence of TMV (Goelet *et al.* 1982). Uracil containing single-stranded template of pDL3 was obtained by coinfection of plasmid with phage M13K07 in the *Escherichia coli* strain CJ236 (Vieira and Messing 1987).

Colony hybridization was used to screen mutagenized colonies for the presence of the desired mismatch (Grunstein and Wallis 1979). The same oligomers used for mutagenesis were used in the colony hybridizations. Colonies that tested positive by hybridization were further confirmed for the desired mutation by double-stranded DNA sequencing (Zagursky *et al.* 1985). In addition, to ensure that no other changes had occurred during mutagenesis, the entire *Cla*I (5,664 bp) to *Nsi*I (6,207 bp) fragment, containing the complete coat protein gene, was sequenced for each clone.

Assembly of mutants. Once sequenced, the *Cla*I to *Nsi*I fragments containing each specific mutation were ligated back into previously unmutagenized *Cla*I to *Nsi*I digested pDL3. Appropriate control ligations of vector and insert were performed to ensure that the sequenced, mutagenized fragment was placed back into an unmutagenized pDL3 vector. An *Nco*I (5,460 bp) to *Spl*I (6,245 bp) fragment from each of the reassembled pDL3 vectors was then ligated into similarly cut full-length pTMV204. Each restriction site used in the ligations was recut to ensure maintenance of the sequence.

***In vitro* transcription and RNA sequencing.** *In vitro* transcriptions of full-length clones were performed as previously described (Ahlquist and Janda 1984; Dawson *et al.* 1986). Transcripts were inoculated onto *N. sylvestris* (N′). Single lesion transfers were made from *N. sylvestris* to *N. tabacum* L. 'Xanthi.' After 10 days virions were purified from systemically infected leaves, and virion RNA was extracted as previously described (Knorr and Dawson 1988) and then sequenced (Zimmern and Kaesberg 1978).

RESULTS

Mutagenesis. Second-strand synthesis reactions yielded between 25 and 150 transformants per mutagenesis experiment. Colony hybridizations indicated that the efficiency of the site-directed mutagenesis was between 16 and 44% for the four oligomers used. All of the transformants selected as positive by colony hybridization also sequenced positive for the desired mutation. Sequence analysis of each mutagenized fragment used in the assembly of full-length TMV clones showed no alterations from the original U1 sequence except for the selected mutation. The four full-length TMV coat protein mutant clones and their subsequent amino acid substitutions are listed in Table 1. These changes are located at several positions throughout the coat protein (Fig. 1).

Symptomatology of mutants. Symptomatology was observed under greenhouse conditions, and appearance of symptoms is indicated as days after inoculation. Wild-type TMV 204 produced a systemic mosaic response in 5–7 days when inoculated onto *N. sylvestris*. Transcripts from each of the four full-length mutant clones were infectious and produced local lesions on *N. sylvestris*. However, local lesion phenotypes on *N. sylvestris* varied among the mutants. Mutant TMV 11 (Fig. 2A) induced lesions that characteristically first showed cell collapse at the point of infection on day four, followed by the appearance of necrotic lesions 4–5 mm in diameter on day five. Necrosis induced by this mutant often spread out along leaf veins near the site of infection, leading to the collapse of large portions of the leaf. Mutants TMV 20 (Fig. 2B) and TMV 25 (Fig. 2C) induced phenotypically identical lesions on *N. sylvestris*. These two mutants produced distinct necrotic spots about 1–2 mm in diameter on day three that did not spread out from the point of infection. Mutant TMV 46 (Fig. 2D) produced lesions that showed both cell collapse and necrosis on day four. Lesions produced by TMV 46 were 1–2 mm in diameter and only occasionally moved out from the original point of necrosis. All four mutants produced lesions identical to those of the parental virus,

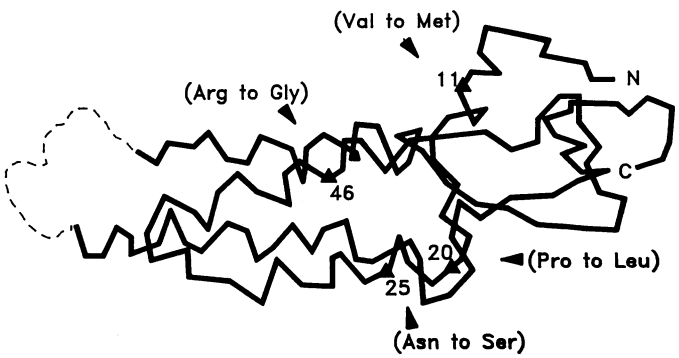

Fig. 1. Diagrammatic representation of the structural configuration of the tobacco mosaic virus coat protein subunit (Bloomer *et al.* 1978), showing the approximate locations of the amino acid substitutions produced by four point mutations.

Table 1. Tobacco mosaic virus coat protein point mutations[a]

Virus	Position[b]	Base change	Amino acid substitution	Symptomatology	
				N. sylvestris	Xanthi
TMV 204	None	None	None	Mosaic	Mosaic
TMV 11	5745	G to A	Val to Met	HR[c]	Mosaic
TMV 20	5773	C to U	Pro to Leu	HR	Mosaic
TMV 25	5788	A to G	Asn to Ser	HR	Mosaic/necrosis
TMV 46	5850	A to G	Arg to Gly	HR	Mosaic/necrosis

[a]The coat protein point mutations were created via site-directed mutagenesis.
[b]Nucleotide number of altered base (Goelet *et al.* 1982).
[c]Hypersensitive reaction.

TMV 204, when inoculated onto the assay host, *N. tabacum* 'Xanthi-nc.'

When infected with the parental virus TMV 204, *N. tabacum* 'Xanthi' characteristically allows the systemic spread of the virus, resulting in a mosaic symptom. The four mutants were also able to move systemically in Xanthi; however, two of the mutants produced distinctly different symptoms. Mutant TMV 25 produced systemic necrosis and leaf deformation on uninoculated systemically infected leaves. Mutant TMV 46 produced limited chlorosis and necrosis in the areas surrounding the veins of systemically infected leaves. Mutants TMV 11 and TMV 20 produced a systemic mosaic similar to that produced by wild-type TMV 204. However, mutant TMV 11 produced some necrosis on inoculated and lower systemically infected leaves. Thus, the coat protein gene mutations also affected the systemic symptoms induced in Xanthi tobacco.

In addition, symptoms were observed in *N. glauca* Graham, *Physalis floridana* Rydb., and *Lycopersicon esculentum* Mill. 'Ace' inoculated with the four mutants. The symptoms observed on these hosts were identical to those produced by the parental virus TMV 204.

Stability of mutants. The stability of the mutants was determined through both maintenance of phenotype and direct sequencing of virion RNA. The distinct phenotype of each mutant was maintained through at least three

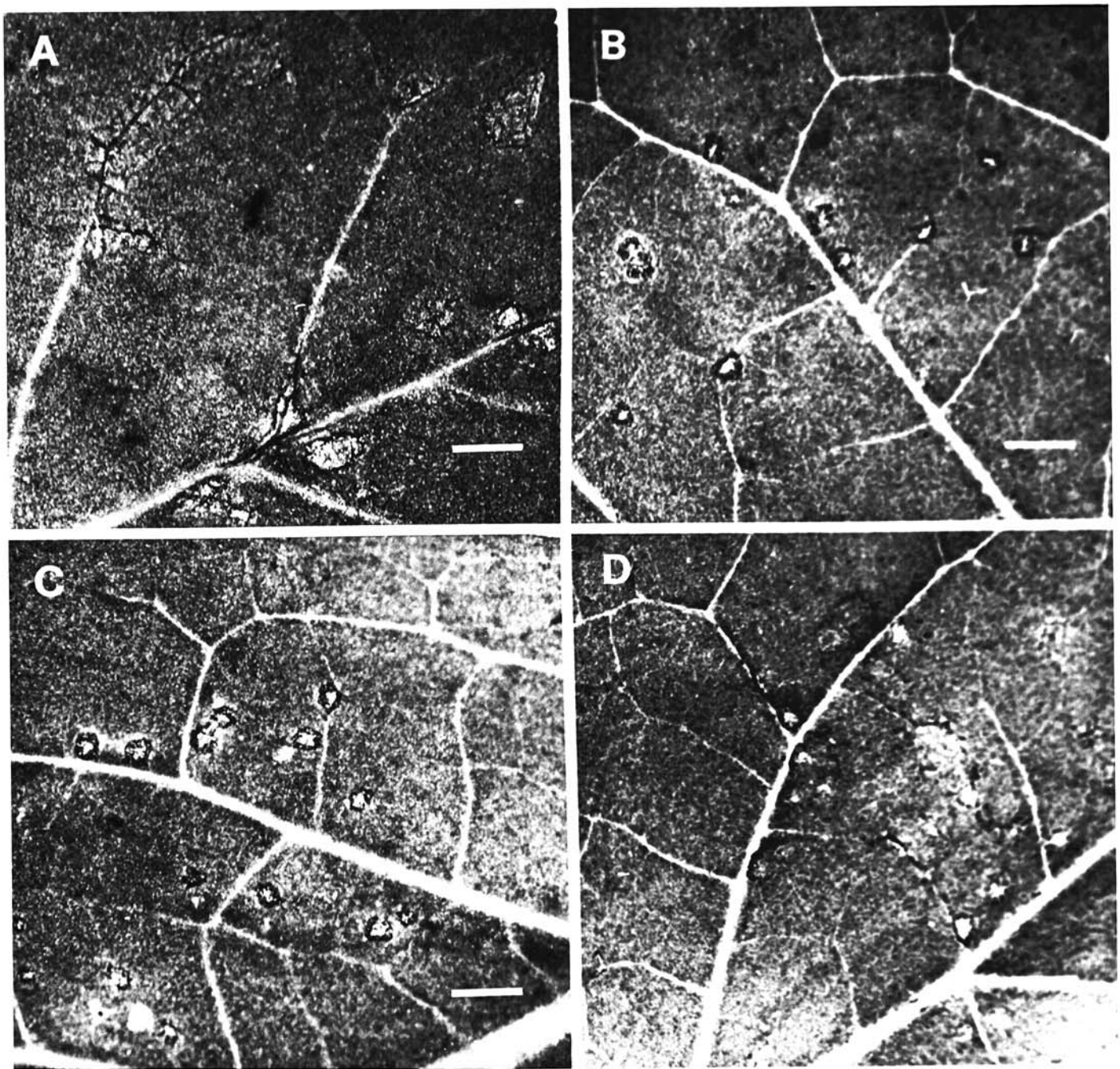

Fig. 2. Local lesions on leaves of *Nicotiana sylvestris* inoculated with tobacco mosaic virus (TMV) coat protein mutants: **A,** TMV 11, 5 days after inoculation; **B,** TMV 20, 3 days after inoculation; **C,** TMV 25, 3 days after inoculation; **D,** TMV 46, 4 days after inoculation. Bars represent 4 mm.

consecutive local lesion passes on *N. sylvestris*. Results of virion RNA sequencing confirmed the stable maintenance of each mutation after a single lesion passage from infectious transcript to systemic host. In addition, the RNA sequence showed no other variations from the wild-type U1 sequence in a 50 base area immediately around each mutation.

DISCUSSION

This study demonstrates that a number of TMV coat protein gene point mutations can alter virus-host interactions. Previous work by Knorr and Dawson (1988) identified an additional coat protein mutation responsible for the induction of HR on *N. sylvestris*. This mutation, at base pair 6,157 (cytosine to uracil), led to the substitution of phenylalanine for serine at amino acid position 148. These point mutations confirm earlier work by Funatsu and Fraenkel-Conrat (1964) and Wittmann and Wittmann-Liebold (1966) that suggested a possible role for the coat protein in the induction of HR on *N. sylvestris*. These earlier studies, directed at deciphering the genetic code, were unable to assign specific coat protein mutations to alterations in host response. However, current advances in technology have allowed us to reexamine this class of mutants and to make precise assignments of sequence to function.

At this time we cannot confirm protein as the active molecule for the induction of HR. It is possible that the altered viral RNA plays this role.

The variations in host responses (local lesions on *N. sylvestris* and systemic symptoms on *N. tabacum* 'Xanthi') observed for the four point mutations were an unexpected result. Similar differences in N′ local lesion phenotypes have previously been observed among various strains of TMV (Fraser 1983). In this study different lesion phenotypes were correlated with the thermal stability of the coat protein subunits. Altered thermal stability indicated potential coat protein structural differences among the strains of TMV. Therefore, one explanation for differences in local lesion phenotypes among mutants is that each mutant has its own structural configuration that has a specific affinity for interacting with a host recognition factor(s). Thus, the ability of a host to mount a resistance response, such as HR, may be directly linked to the efficiency with which the host recognizes a specific virus product.

The point mutations created resulted in widely diverse amino acid substitutions at different positions throughout the coat protein molecule (Fig. 1). However, the point mutations did not prevent the assembly of the virus. Mutant coat protein sequences were subjected to computer analysis for several kinds of protein secondary structure (Devereux *et al.* 1984). Each mutant coat protein showed changes in predicted secondary structure when compared to the wild-type TMV 204 coat protein sequence. However, the predicted changes differed for each of the four mutants. Thus, at this level of computer analysis, no single alteration in coat protein structure explained the ability of these mutations to alter host response. This suggests that several coat protein structural or conformational configurations may result in recognition by host factor(s) and induction of HR. Indeed, previous work in our laboratory (Dawson *et al.* 1988) has shown that large deletions in the TMV coat protein gene can also induce necrosis in *N. sylvestris*. In addition, rates of TMV mutations resulting in mutants that induce HR are significantly higher (10^{-3}) than rates of revertant mutations resulting in mutants that cause systemic symptoms (Aldaoud 1987). This suggests that the TMV wild-type coat protein gene may be a rare sequence responsible for systemic disease in *N. sylvestris*.

Although all four of our mutants induced HR on *N. sylvestris*, Funatsu and Fraenkel-Conrat (1964) listed other coat protein amino acid substitutions that did not induce HR. Therefore, it should be stressed that not every possible nucleotide substitution in the coat protein will lead to the induction of HR. Thus, it seems likely that only specific nucleotide changes that result in specific alterations of the wild-type coat protein sequence will lead to the induction of the host resistance response.

ACKNOWLEDGMENTS

This research was funded in part by grants 87-CRCR-1-12450 from the U.S. Department of Agriculture and I-882-85 from the U.S.-Israel Binational Agricultural Research and Development Fund (BARD).

LITERATURE CITED

Ahlquist, P., and Janda, M. 1984. cDNA cloning and *in vitro* transcription of the complete brome mosaic virus genome. Mol. Cell. Biol. 4:2876-2882.

Aldaoud, R. 1987. Biological characteristics, variations, and factors affecting variation in cloned tobacco mosaic virus. Ph.D. thesis, University of California, Riverside.

Bloomer, A. C., Champness, J. N., Bricogne, G., Staden, R., and Klug, A. 1978. Protein disk of tobacco mosaic virus at 2.8 Å resolution showing the interactions within and between subunits. Nature 276:362-368.

Dawson, W. O., Beck, D. L., Knorr, D. A., and Grantham, G. L. 1986. cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. Proc. Natl. Acad. Sci. USA 83:1832-1836.

Dawson, W. O., Bubrick, P., and Grantham, G. L. 1988. Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. Phytopathology 78:783-789.

Devereux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387-395.

Flor, H. H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9:275-296.

Fraser, R. S. S. 1983. Varying effectiveness of the N′ gene for resistance to tobacco mosaic virus in tobacco infected with virus strains differing in coat protein properties. Physiol. Plant Pathol. 22:109-119.

Funatsu, G., and Fraenkel-Conrat, H. 1964. Location of amino acid exchanges in chemically evoked mutants of tobacco mosaic virus. Biochemistry 3:1356-1361.

Geisselsoder, J., Witney, F., and Yuckenberg, P. 1987. Efficient site-directed *in vitro* mutagenesis. BioTechniques 5:786-791.

Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J., and Karn, J. 1982. Nucleotide sequence of tobacco mosaic virus RNA. Proc. Natl. Acad. Sci. USA 79:5818-5822.

Grunstein, M., and Wallis, J. 1979. Colony hybridization. Methods Enzymol. 68:379-389.

Keen, N. T., and Staskawicz, B. 1988. Host range determinants in plant pathogens and symbionts. Annu. Rev. Microbiol. 42:421-440.

Knorr, D. A., and Dawson, W. O. 1988. A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*. Proc. Natl. Acad. Sci. USA 85:170-174.

Legrand, M., Fritig, B., and Hirth, L. 1976. Enzymes of the phenylpropanoid pathway and the necrotic reaction of hypersensitive tobacco to tobacco mosaic virus. Phytochemistry 15:1353-1359.

Meshi, T., Ishikawa, M., Motoyoshi, F., Semba, K., and Okada, Y. 1986. *In vitro* transcription of infectious RNAs from full-length cDNAs of tobacco mosaic virus. Proc. Natl. Acad. Sci. USA 83:5043-5047.

Russell, G. E. 1978. Pages 231-264 in: Plant Breeding for Pest and Disease Resistance. Butterworth Publications, Boston.

Saito, T., Meshi, T., Takamatsu, N., and Okada, Y. 1987. Coat protein

gene sequence of tobacco mosaic virus encodes host response determinant. Proc. Natl. Acad. Sci. USA 84:6074-6077.
Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.
Wittmann, H. G., and Wittmann-Liebold, B. 1966. Protein chemical studies of two RNA viruses and their mutants. Cold Spring Harbor Symp. Quant. Biol. 31:163-172.
Zagursky, R. J., Baumeister, K., Lomax, N., and Berman, M. L. 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gene Anal. Technol. 2:89-94.
Zimmern, D., and Kaesberg, P. 1978. 3′-Terminal nucleotide sequence of encephalomyocarditis virus RNA determined by reverse transcriptase and chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 75:4257-4261.