AN ABSTRACT OF THE THESIS OF

John A. Lindbo for the degree of Doctor of Philosophy in Microbiology presented on August 19, 1993.

Title: Virus Resistance in Transgenic Plants Expressing Translatable and Untranslatable Forms of the Tobacco Etch Virus Coat Protein Gene Sequence

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Dr. William G. Dougherty

Tobacco etch virus (TEV) is a member of the *Potyvirus* genus, one of the largest and most economically important groups of plant viruses. In an attempt to generate TEV-resistant transgenic plants, I have applied the concept of pathogen derived resistance (PDR). The concept of PDR proposes that pathogen resistance genes can be derived from a pathogen's own genetic material. To investigate this hypothesis, transgenic *Nicotiana tabacum* plants expressing one of several different forms of the TEV coat protein (CP) gene sequence were generated. Transgenic plants were screened both for transgene expression and TEV-resistance. The particular TEV-resistance phenotype observed varied with the particular form of the TEV CP gene expressed by the plant.

Transgenic plant lines expressing untranslatable sense-stranded transcripts often showed complete resistance to TEV. Protoplasts from these TEV-resistant plant lines would not support TEV replication. In this group of

transgenic plants steady state transgene RNA levels did not correlate with virus resistance.

Plant lines accumulating either full length or truncated forms of CP displayed different resistance phenotypes, depending upon the particular form of CP which accumulated in the plant. Some of the resistance phenotypes observed in these TEV-challenged transgenic plant lines were: (1) a delay in the appearance of TEV-induced symptoms; (2) an attenuation of TEV-induced symptoms; or (3) the ability to recover from (or outgrow) TEV infection. Whole plant inoculation experiments were used to demonstrate that TEV-recovered transgenic plant tissue could not be infected with TEV. Protoplast experiments demonstrated that TEV-recovered transgenic plant tissue would not support TEV replication. TEV-recovered transgenic plant tissue was, however, susceptible to the related potyvirus potato virus Y. Interestingly, transgene RNA and protein levels were reduced 10-20 fold in TEV-recovered tissue. It is proposed that the TEV-resistant state and reduction in transgene RNA levels are mediated by a cytoplasmically-based activity which targets both the transgene RNA and TEV genomic RNA for degradation. Such an activity could be functioning in seemingly unrelated biological phenomenon such as cross protection and some examples of sense- (or co-) suppression of genes.

VIRUS RESISTANCE IN TRANSGENIC PLANTS EXPRESSING TRANSLATABLE AND UNTRANSLATABLE FORMS OF THE TOBACCO ETCH VIRUS COAT PROTEIN GENE SEQUENCE

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PREFACE

In chapter two of this thesis, Laura Silva-Rosales and William G. Dougherty are co-authors. L. Silva-Rosales assisted in the literature search, organization of Figure II.1, Tables II.1 - II.4 and in the organization of the text. W. Dougherty assisted in general organization and content of the text and figures.

In chapter five of this thesis, Laura Silva-Rosales, William Proebsting and William G. Dougherty are co-authors. L. Silva-Rosales generated the results shown in Table V.1. W. Proebsting and W. Dougherty performed the grafting studies. W. Dougherty also generated the data presented in Table V.2.

VIRUS RESISTANCE IN TRANSGENIC PLANTS EXPRESSING TRANSLATABLE AND UNTRANSLATABLE FORMS OF THE TOBACCO ETCH VIRUS COAT PROTEIN GENE SEQUENCE

CHAPTER I

POTYVIRUSES

Introduction

Characteristics of the *Potyviridae*

The *Potyviridae* family of plant viruses consists of a large number of plant viral pathogens which share common structural and biological properties. Structurally, members of this virus family are flexuous rod-shaped viruses. Virion particles are composed of a single stranded RNA of plus (or message) sense polarity, helically encapsidated by multiple copies of a single coat protein monomer. The RNA genome is covalently linked to a virus-encoded protein (VPg) at its 5' end and has a 3' polyadenylate sequence (review, Riechmann *et al.*, 1992). All members of the *Potyviridae* induce the formation of characteristic cylindrical or pinwheel-shaped inclusion bodies in the cytoplasm of infected plant cells (Edwardson and Christie, 1991) . Infections by certain members of the *Potyviridae* also results in the formation of nuclear inclusion and/or amorphous cytoplasmic inclusion bodies (Edwardson and Christie, 1991).

Organization of the *Potyviridae*

Several taxonomic methods have been proposed for the *Potyviridae* based on host range, ability to cross protect, inclusion body morphology, coat protein amino acid sequence homology, nucleotide sequence homology, or serological relationships. However, with the limited number of *Potyviridae* examined and the continuum of variation observed, it has been difficult to arrive at a consensus which adequately distinguishes a "strain" from a "virus". As such, controversy still remains regarding their taxonomic status. It has been recently suggested that the *Potyviridae* be divided into three genera (Barnett, 1992), based upon their natural vectors:

Genus 1: Aphid-transmitted members (*Potyvirus*).

Genus 2: Fungus-transmitted members (*Bymovirus*).

Genus 3: Mite-transmitted members (*Rymovirus*).

This introduction will focus on members of the aphid-transmitted *Potyvirus* genus, especially tobacco etch virus (TEV).

Transmission

Potyviruses are transmitted by aphids in a non-persistent, non-circulative (stylet-borne) manner (review, Pirone, 1991). Aphids can acquire virus by probing infected tissue for only a few seconds, retain the ability to transmit virus for several hours, and can transmit virus to healthy plants with short probes lasting less than one minute. Because aphids do not retain the ability to transmit virus for extended periods of time, in field conditions virus usually comes from nearby inoculum sources. A typical potyvirus is often transmissible by several different aphid species. Given the promiscuous feeding behavior of aphids and the characteristics of stylet-

borne transmission, potyviruses can be spread in a field by aphids simply passing through, and not colonizing, a crop.

Aphid transmission of potyviruses involves at least two viral encoded proteins: (1) the "helper component" (the 56 kDa HC-PRO in TEV) and (2) the virus capsid protein. Nucleotide sequence comparisons between non-aphid and aphid transmissible virus isolates have been used to predict which amino acids in these two proteins may be important for aphid transmissibility. Infectious transcripts from a full-length cDNA clone of the potyvirus tobacco vein mottling virus (TVMV) have been used to confirm the importance of specific amino acids in both the capsid protein and HC-PRO in aphid transmissibility (review, Pirone, 1991).

Genome structure

The following discussion on genome organization and expression is based largely on experimentation with the potyviruses TEV, TVMV, and plum pox virus (PPV). The TEV system will be used to illustrate proteolytic processing and gene expression. In the following discussion, protein sizes mentioned are for TEV. Other potyviruses will not necessarily have identically-sized proteins.

The RNA genome of potyviruses is a single strand message-sense RNA of about 9,600 nucleotides (nt). It is organized as a single large open reading frame (ORF), encompassing about 95% (>9,100 nt) of the RNA molecule. A viral encoded protein (VPg) is covalently linked to the 5' terminal nucleotide and a polyadenylate sequence is present at the 3' terminus. At least nine gene products are encoded in this large ORF and are initially expressed as a large polyprotein which is proteolytically processed into mature viral protein products (review, Dougherty and Carrington,

1988). The various TEV gene products and their identities/functions are presented in Fig I.1. and discussed below.

Gene expression

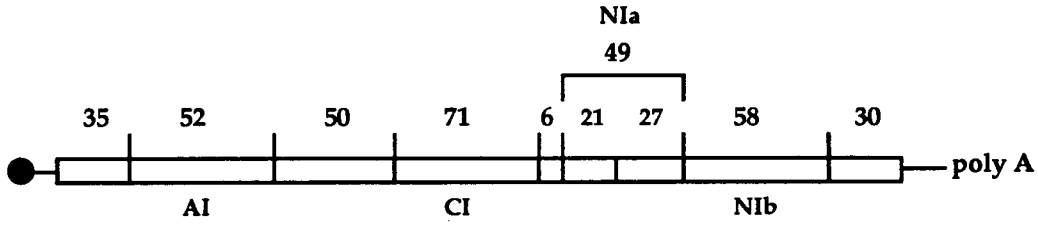
Three distinct proteolytic activities have been associated with TEV gene products. The first identified proteolytic activity was ascribed to the TEV 49 kDa NIa polyprotein (Carrington and Dougherty, 1987), one of the components of the nuclear inclusion body which forms in TEV-infected plant tissue (Dougherty and Hiebert, 1980b). The 49 kDa polyprotein is further processed into two proteins of ca. 21 and 27 kDa (Dougherty and Parks, 1991). The amino-terminal 21 kDa of the NIa polyprotein is the VPg of the virus (Murphy *et al.*, 1990). The proteolytic activity of the NIa polyprotein resides in the carboxy-terminal 27 kDa portion (Dougherty and Parks, 1991). The NIa proteinase of TEV has been identified as a trypsin-like cysteine proteinase similar to the 3C proteinase of picornaviruses (Dougherty et al., 1989). The proteolytic specificities associated with the 27 and 49 kDa proteins appear to be identical in cell-free studies (Dougherty and Parks, 1991). It is uncertain whether the 49 or 27 kDa form of this proteinase is responsible for *in vivo* cleavage of the polyprotein (review, Dougherty and Carrington, 1988).

The TEV NIa proteinase cleaves at a highly conserved site which spans the seven amino acid sequence:

$$\begin{array}{c} \text{Ile} \qquad\qquad \text{Glu} \quad \text{Ser} \\ \text{Glu-Xaa-Leu-Tyr-Xaa-Gln / Gly} \\ \text{Val} \end{array}$$

Figure I.1. Genome organization and protein products of the potyvirus tobacco etch virus (TEV). Sizes of proteins are deduced from predicted amino acid sequence derived from the TEV genomic nucleotide sequence. Other potyviruses have similar, but not identically sized, protein products. In the genome map **(A)**, the solid circle represents the VPg, the thin horizontal lines represent the TEV genome 5' and 3' untranslated sequences and the long open rectangular box denotes the open reading frame. Vertical lines are used to show locations of proteolytic cleavage of the polyprotein. Molecular weights (in kDa) of the various processed TEV gene products are presented above the diagram. Proteins which aggregate to form the various inclusion bodies during infection are identified: AI and CI represent the proteins which form the amorphous cytoplasmic inclusion and cylindrical inclusion bodies, respectively. The NIa polyprotein and NIb protein together make up the nuclear inclusion body co-crystal. **(B)** Predicted protein function and experimentally determined activities (in parenthesis) are listed in the right hand column. The symbol, ??, denotes proteins for which no function is known. The 49 kDa NIa polyprotein is processed into 21 and 27 kDa proteins. The proteolytic activity is associated with the 27 kDa protein. Either the 21 or the 49 kDa protein can function as the VPg moiety.

A



B

GENE PRODUCT		PREDICTED AND DETERMINED FUNCTION
	35 kDa	Proteolytic processing , cell-to-cell movement (N-PRO proteinase)
	52 kDa	Proteolytic processing, insect transmission (helper component, HC-PRO proteinase, AI protein)
	50 kDa	??
	71 kDa	Replication, cell-to-cell movement (helicase, ATPase, CI protein)
	6 kDa	Replication, membrane binding protein
49 kDa (NIa)	21 kDa	Replication (VPg)
	27 kDa	Polyprotein processing, replication (NIa proteinase)
	58 kDa	Replication; putative RNA-dependent RNA-polymerase (NIb protein)
	30 kDa	Encapsidation and insect transmission (capsid protein, insect transmission)

Figure I.1.

Cleavage occurs between the Glu (Gln) and Gly (Ser) residues. Positions represented by Xaa are occupied by neutral or hydrophobic amino acids. In cell-free studies, various amino acids in the Xaa positions affect the rate of cleavage (Dougherty *et al.*, 1989; Dougherty and Parks, 1989). Cleavage sites composed of strictly and loosely conserved amino acids have been found in other potyviruses also.

The two other proteolytic activities described for TEV have only been demonstrated to function in a mono-molecular fashion (i.e. *cis*). The 52 kDa protein, referred to as HC-PRO, is a papain-like cysteine proteinase (Oh and Carrington, 1989) which cleaves at its carboxy-terminal end to release itself from the polyprotein. Cleavage is between a Gly/Gly dipeptide (Carrington *et al.*, 1989). A recently described proteolytic activity involves the 35 kDa protein (P1) of TEV (Carrington *et al.*, 1990; Verchot *et al.*, 1991). This proteinase, located at the N-terminus of the genome derived polyprotein, also cleaves at its carboxy-terminus to release itself from the polyprotein. Sequence homology and mutagenesis studies suggest the P1 proteinase resembles cellular serine-type proteinases (Verchot *et al.*, 1991, 1992). In TEV the P1 catalyzed cleavage is between a Tyr-Ser dipeptide (Verchot *et al.*, 1992).

Gene regulation

It has been proposed that potyviruses may regulate their gene expression at a post-translational level via differential processing rates at various cleavage sites. Potyviruses may also use differential protein stabilities and/or the shunting of proteins into inclusion bodies as a means of regulating gene activity in a post-translational manner. A proposed

pathway of proteolytic processing in potyviruses is presented in schematic form in Fig I.2.

Replication

Because of their genome structure and gene expression mechanism, potyviruses have been placed in the super group of "picorna-like" viruses (review, Goldbach, 1987). As such, it is likely potyvirus replication will be similar to picornavirus replication. Replication of picornaviruses occurs in the cytoplasm of infected cells and involves a membrane bound replicase complex. The picornaviral proteins 2B, 2C, 3A, 3C and 3D are involved in replication (review, Kuhn and Wimmer, 1987). Sequence homology studies have revealed regions of homology between picornavirus 2C, 3C, and 3D proteins and the 71, 27 and 58 kDa proteins of TEV (review, Goldbach, 1990). Structural and functional similarities have been observed between picornavirus and potyvirus proteins as well. On a functional level, the picornavirus 2BC protein and potyvirus 71 kDa protein both induce the formation of smooth walled vesicles in the cytoplasms of infected cells (Calder and Ingerfeld, 1990). The picornavirus 3A and TEV 6 kDa proteins do not show sequence homology, though they appear to be biochemically similar in that both are small proteins with hydrophobic core sequences. The 3A protein is a component of the picornavirus replicase complex (review, Kuhn and Wimmer, 1987). The 3B and 21kDa proteins both function as VPgs (although in a portion of TEV virions, a 49 kDa polyprotein is linked to the TEV genome) (Murphy *et al.*, 1990). The 3C and 27 kDa proteins both have proteolytic activities, and the 3D and 58 kDa proteins both have amino acid sequences conserved among RNA-dependent RNA-polymerases. Helicase and ATPase activities have recently

Fig I.2. Putative proteolytic processing scheme of the potyvirus tobacco etch virus (TEV). Shown is a map of the TEV genome (see Fig I.1 for details), its translation products, and a proposed scheme of proteolytic processing. The solid circle in the genome map represents the VPg. Proteolytic activities have been mapped to three proteins in TEV: (1) the 35 kDa protein, (2) the carboxy-terminal half of the 52 kDa HC-PRO, and (3) the 27 kDa NIa protein. Those regions with identified proteolytic activities are shaded. Arrows indicate the proteinase responsible for cleavage at the dipeptide sequences (single letter amino acid code) indicated. A series of autocatalytic events occur rapidly (above dotted line in diagram) and possibly co-translationally. Subsequent cleavage reactions (below the dotted line) may be mono- or bi-molecular. The 49 kDa protein is autocatalytically processed at a Glu-Gly dipeptide to form a 21 and 27 kDa sized protein. The 27 kDa portion retains proteolytic activity.

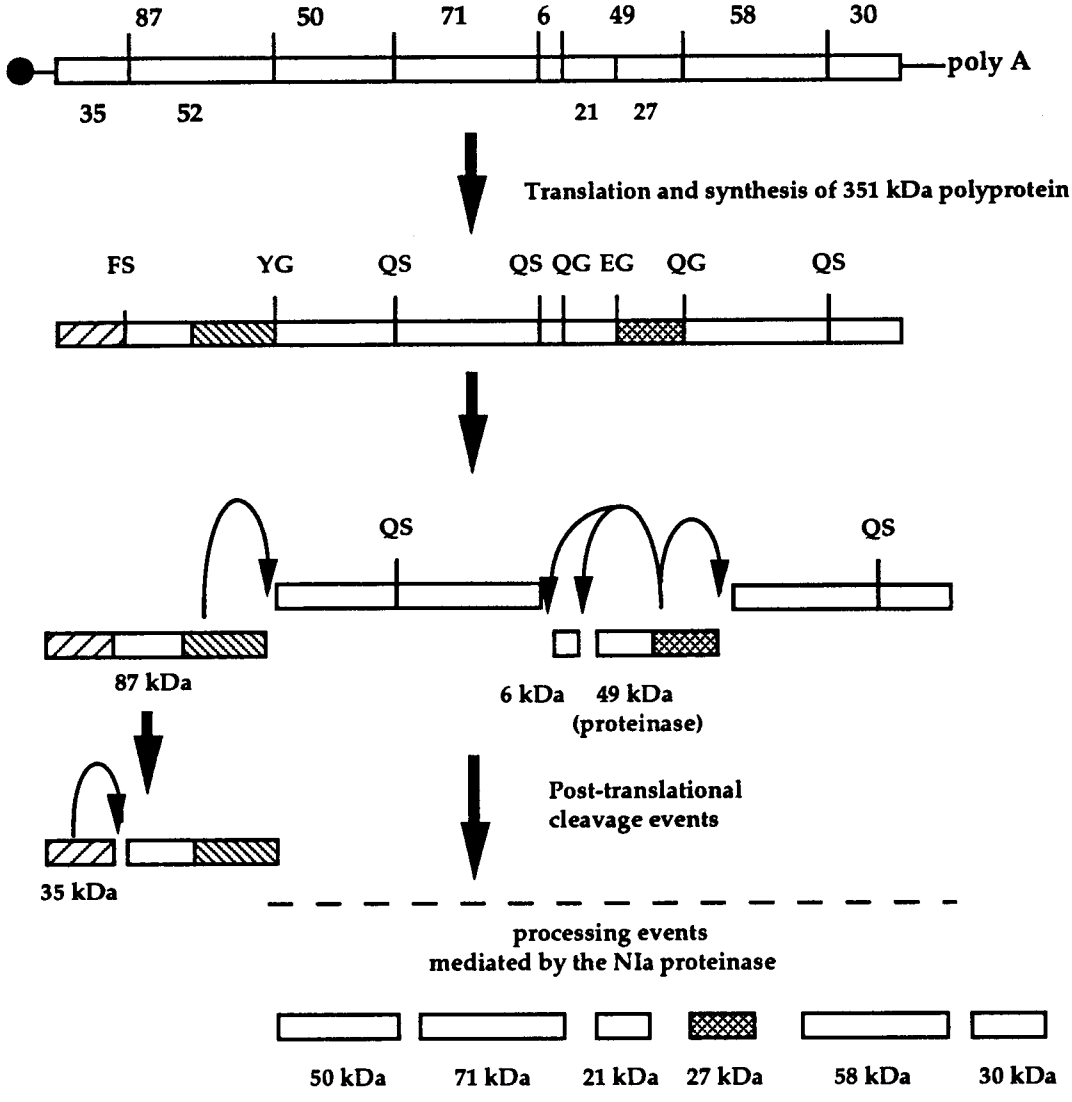

87
50
71
6
49
58
30
poly A
35
52
21
27
Translation and synthesis of 351 kDa polyprotein
FS
YG
QS
QS QG EG
QG
QS
QS
QS
87 kDa
6 kDa
49 kDa
(proteinase)
Post-translational
cleavage events
35 kDa
processing events
mediated by the NIa proteinase
50 kDa
71 kDa
21 kDa
27 kDa
58 kDa
30 kDa

Figure I.2

been demonstrated for the PPV 71 kDa protein *in vitro* (Laín *et al.*, 1990, 1991) implicating this protein in virus replication. A completely functional "replicase complex" has not been isolated from potyvirus infected tissue.

This discussion has briefly touched on several of the nine identified gene products encoded by the TEV genome. Activities and roles in the viral replication cycle have been described, but do not preclude other functions associated with partially processed polyproteins containing these gene products. A putative function(s) for the TEV 50 kDa protein product has not been proposed.

Virus-host relationship

The formation of large distinctive inclusion bodies is a hallmark of potyvirus-infected tissue. Potyvirus infected cells contain cylindrical or pinwheel shaped cytoplasmic inclusion (CI) bodies comprised of viral encoded protein. Initially the CI's are associated with the infected cell membrane and later are found free in the cytoplasm. The CI protein has helicase and ATPase activities (Laín *et al.*, 1990, 1991). These cytological and biochemical observations have lead to speculation that the CI inclusion protein may function both in virus replication and cell-to-cell movement. Sequence homology studies suggest that the 35 kDa proteinase may also be involved in movement of virus infection from cell-to-cell within a plant. Limited sequence homology has been detected between the TVMV 35 kDa proteinase and the TMV 30 kDa movement protein (Domier *et al.*, 1987).

A few potyviruses also form nuclear inclusions (NI) and/or amorphous cytoplasmic inclusions (AI). NI bodies are distinctively shaped crystals composed of two virus-encoded proteins, the 49 kDa polyprotein (21 kDa VPg and 27 kDa proteinase) and the putative RNA-dependent-RNA

polymerase of ca. 58 kDa. The AI, when formed, is an aggregate of the HC-PRO protein. In addition to its proteolytic function, HC-PRO is also required for aphid transmission. The relationship between these two functions is not understood. All potyvirus-induced inclusions can be readily viewed in stained epidermal strips by light microscopy. Purification schemes to obtain inclusion bodies also have been established.

On a whole plant level, systemic symptoms usually appear within 6-8 days post inoculation (mechanically- or aphid-vectored inoculum). Symptoms on systemically infected leaves often include mosaic and mottling, vein clearing, chlorosis, streaking, necrosis, leaf distortion, etc.

Tens of milligrams of virions can usually be obtained from a kilogram of infected plant tissue. Such purified virus preparations are often used to generate antibodies to potyvirus coat proteins for use in serological based detection schemes (enzyme-linked immunosorbent assay, etc.). Recent serological and structural analysis of potyvirus coat proteins has revealed that the amino- and carboxy-termini of the coat protein reside on the exterior of the virion (Allison *et al.*, 1985a; Shukla and Ward, 1989). These termini greatly differ in both sequence and length among potyviruses. The internal amino acids of the coat protein, however, are relatively conserved between potyviruses. Antibodies directed toward the coat protein amino- or carboxy-terminus can be virus specific, whereas antibodies directed toward the internal, conserved amino acid residues are usually cross-reactive with different potyviruses (Dougherty *et al.*, 1985).

Economic importance

Several factors contribute to potyviruses being an economically important virus group, including the following: (1) Although the host range of most potyviruses is narrow, there is virtually no agricultural crop which one or more potyviruses cannot infect. (2) Symptoms on infected plants are often quite visible. Vein clearing, leaf chlorosis, necrosis, leaf distortion, stunting, and distorted fruits and seeds can result in considerable crop losses in yield and/or quality. (3) Potyviruses are readily transmitted from infected to healthy plants. Aphid transmitted potyviruses are transmitted in a non-persistent, non-circulative (stylet-borne) manner. Aphids can acquire and transmit virus with short feeding probes lasting only a few seconds. (4) Potyviruses are found in a wide range of environments. They are especially prevalent in sub-tropical or tropical regions where virus can overwinter in weeds, alternate crops, or volunteer plants. (Though some potyviruses may also enter a field via contaminated seed). Tropical, sub-tropical and temperate climates also support insect populations which may vector the virus.

The economic impact of the group as a whole is difficult to estimate. The extent of crop loss depends upon a complex interaction of growing conditions, cultivars, virus strains, cultural practices, time and extent of infection in the field, etc. Chronic losses due to viral infection may often go undetected, but epidemics can cause complete crop loss. Financial investments in virus control measures such as resistance breeding programs and seed certification programs also represent one aspect of the economic impact of potyviruses.

Control schemes

In addition to cultural practices to remove primary sources of inoculum, seed certification and traditional breeding programs have been implemented to control potyviruses. Resistance phenotypes are often inherited in a recessive manner and therefore difficult to identify (Provvidenti and Hampton, 1992). Also, resistant germplasm is often not available for every crop and viruses rapidly mutate to overcome deployed resistance genes.

Because of their economic importance and the inaccessibility or ineffectiveness of many naturally occuring virus resistance genes, it is of interest to develop alternative, effective means of virus control. Virus resistant plants have been genetically engineered by deriving resistance genes from the virus itself (reviews, Beachy *et al.*, 1990; Gadani *et al.*, 1990). It has been demonstrated that transgenic host plants which express the coat protein nucleotide sequence of a particular plant virus are resistant to subsequent infection by that virus. Although the mechanism of this resistance is not entirely understood, this approach has been applied, with varying degrees of sucess, to a wide variety of plant viruses, including potyviruses (review, Lindbo *et al.*, 1993).

Conclusions

Potyviruses are a very large group of plant viruses which can have significant economic impact on a wide variety of agriculturally important crops. This work was initiated to examine genetically engineered resistance to potyviruses. At the onset of this work no plant species had yet been genetically engineered for resistance to a potyvirus. The specific goals were to develop a model (transgenic) plant-potyvirus system with which to

examine "genetically engineered resistance". Experiments were conducted to ask several questions:

Are transgenic plants which express the coat protein (CP) gene of TEV resistant to TEV?

Are transgenic plants which accumulate truncated forms of the TEV CP resistant to TEV?

Is virus resistance due to the CP or its RNA?

If resistance is found, what is the mechanism of resistance?

To address these questions the well studied potyvirus TEV and a TEV-susceptible commercially grown tobacco cultivar (*Nicotiana tabacum* cv. B49) were selected as a model system. The following chapters summarize the current status of genetically engineered resistance to potyviruses, and describe in detail my efforts to develop a transgenic plant system with which to examine this phenomenon. A series of transgenic plant lines have been generated, screened for transgene expression at the RNA and protein level and assayed for virus resistance phenotypes. Experiments were also performed to examine virus resistance at the cellular level to determine if resistance was due to an inhibition of virus replication, or virus movement. These studies have provided data which suggests an unexpected phenomenon is at work which may provide insight into aspects of basic gene regulation, and virus resistance strategies.

CHAPTER II

PATHOGEN DERIVED RESISTANCE TO POTYVIRUSES: WORKING, BUT WHY?

Abstract

The theory of pathogen derived resistance suggests that pathogen resistance genes can be developed from a pathogen's own genetic material. For example, transgenic plants expressing plant virus coat protein genes are often virus resistant. Non-structural virus genes also have been expressed in plants, with mixed results. Recent experiments suggest that, at least in some cases, resistance may be RNA mediated. In all cases the exact mechanism of this resistance remains a mystery. This review discusses examples of pathogen derived resistance for members of the *Potyviridae* family of plant viruses and offers some possible mechanisms to account for this intriguing phenomenon.

Introduction

The *Potyviridae* is a large, agronomically important family of plant viruses which share common structural, biochemical, and biological properties. The family is composed of three genera, the *Potyvirus* genus containing the most members (Barnett, 1992). Potyviruses are flexuous rod-shaped plant viruses approximately 12-15 nm in diameter and 700 to 900 nm in length. Virions are composed of a single-strand of plus sense RNA nearly 10,000 nucleotides in length, encapsidated by approximately 2000 copies of a coat protein (CP) monomer. The RNA genome is covalently linked to a virus-encoded protein (VPg) at its 5' end and has a 3' polyadenylate sequence. A potyvirus genome contains a single open reading frame encompassing over 95% of the genome (Fig. II.1.). All virus-encoded proteins are expressed initially as a large polyprotein that is proteolytically processed into mature viral products. No subgenomic RNAs are produced during the replication cycle (Dougherty, 1983).

Potyviruses are significant agricultural pathogens for several reasons. First, there are over 100 members in this genus. Although each individual member has a fairly limited host range, the large number of potyviruses insures that few, if any, agricultural crop species cannot be infected by one or more members of this genus. Second, potyviruses are transmitted by aphids in a non-persistent, non-circulative (stylet borne) manner. This mode of transmission enables aphids to efficiently vector the virus and makes control of potyvirus transmission difficult (review, Pirone, 1991). Finally, potyviral infection can result in necrosis, chlorosis, and/or stunting of the infected plant, adversely affecting the quantity and quality of the leaves, fruits, or seeds produced.

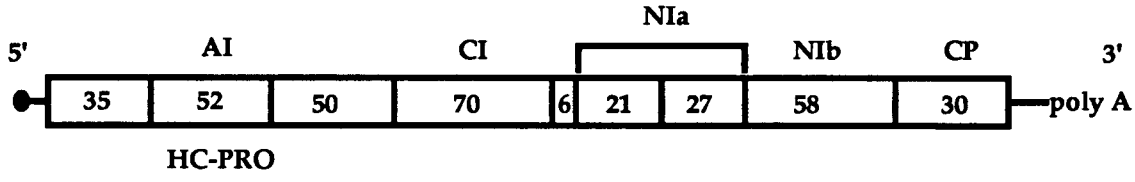


Figure II.1. Schematic representation of the genome organization and protein products of tobacco etch virus (TEV), a typical potyvirus. In the TEV genome map, the solid circle represents the VPg; the thin horizontal lines represent the TEV genomic 5' and 3' untranslated sequences; and the long rectangular box represents the open reading frame. Vertical lines dissecting the box are used to show locations of proteolytic cleavage of the polyprotein. Approximate sizes of proteins (in kilodaltons) are listed in the open boxes. Selected protein identities are shown above or below the corresponding box. Abbreviations used are as follows: HC-PRO, helper component-proteinase; AI, amorphous inclusion protein; CI, cylindrical inclusion protein; NIa, nuclear inclusion body protein a; NIb, nuclear inclusion body protein b; CP, coat protein. Other potyviruses have similar but not identically sized proteins.

Because plant viruses can have devastating effects on marketable crop yield, a significant amount of effort has been devoted to plant virus control. Using virus resistant cultivars is an effective way to control plant viruses. Traditional breeding practices have been successful in incorporating potyvirus resistance genes into commercially important cultivars (Provvidenti and Hampton, 1992). However, virus resistant germplasm can be unavailable or difficult to identify. Additionally, a continually mutating virus population often renders resistance genes ineffective after a few growing seasons.

The theory of pathogen derived resistance (PDR) (Sanford and Johnston, 1985) proposed that virus resistance genes may be developed from a virus' own genetic material. PDR has predicted that selected genes, when removed from a virus, inserted into and expressed from a host plant genome, may render that plant resistant to virus infection. Such "genetic engineering" offers the potential to expand the repertoire of resistance genes which can then be deployed through traditional breeding methods. In 1986, it was first demonstrated that transgenic plants which express the tobacco mosaic virus (TMV) CP were resistant to TMV (Powell-Abel *et al.*, 1986). This phenomenon has since been tested for a wide variety of plant virus groups (Beachy *et al.*, 1990; Gadani *et al.*, 1990) and is often referred to as "coat protein meditated resistance" (CPMR).

To date, several examples of "genetically engineered" potyvirus resistance have been demonstrated. This review chapter will summarize the various results obtained and speculate on possible mechanisms of resistance. In this review "virus-specific resistance" refers to those cases where a transgenic plant expressing a viral cistron is resistant to the virus from which the transgene was derived. "Broad spectrum resistance" refers to those cases in

which a transgenic plant is resistant to a virus other than the virus from which the transgene was derived.

Coat protein mediated resistance (CPMR)

The first example of CPMR to a potyvirus was presented by Stark and Beachy (1989). They reported that transgenic tobacco plants expressing the CP of soybean mosaic virus (SMV) were resistant to the potyviruses tobacco etch virus (TEV) and potato virus Y (PVY). Stark and Beachy referred to the ability of a potyvirus CP to confer resistance to a distantly related (heterologous) potyvirus as "broad spectrum" resistance. Shortly thereafter, Lawson and co-workers (Lawson *et al.*, 1990) published the first report of virus-specific CPMR to a potyvirus. Since these two initial reports, numerous examples of both virus-specific and broad spectrum potyvirus CPMR have been described (summarized in Table II.1.). Select examples are discussed in this section.

In the report of Stark and Beachy (1989), transgenic tobacco (*N. tabacum* cv. Xanthi) plants expressing the CP of SMV were challenged with various dilutions of purified preparations of TEV and PVY. Since tobacco is a non-host for SMV, virus-specific CPMR could not be tested with this system. Six transgenic plant lines were analyzed for virus resistance. Transgenic plant line 1052, while not the highest CP accumulating line (SMV CP = 0.15% total soluble protein), was the most resistant of the six lines examined. Resistance was manifested as a delay in the appearance of symptoms and an attenuation of symptoms. To examine the effect of SMV CP levels on protection, plants of line 1052 (heterozygous or homozygous for the CP gene) were generated. Plants of line 1052, homozygous for the CP gene, express 2-3 times more SMV CP than

Table II.1. [a]Abbreviations used are: (high)= high virus inoculum levels; (low)= low virus inoculum levels; S= wild type susceptibility; R= resistance characterized by one or more of the following : delay in appearance of syptoms, symptom attenuation, ability to outgrow infection or a reduction in virus titer; HR= highly resistant--resistance is typified as described in R, only to a greater degree; CR= complete resistance, no detectable symptoms or virus after challenge. Note: R and HR designations are relative and should only be compared when the same transgenic plant line has been inoculated with different viruses.

Other abbreviations used are as follows: SMV, soybean mosaic virus; PVY, potato virus Y; TEV, tobacco etch virus; TVMV, tobacco vein mottling virus; TVBMV, tobacco vein banding mosaic virus; PRV, papaya ringspot virus; PeMV, pepper mottle virus; ZYMV, zucchini yellow mosaic virus; PPV, plum pox virus; WMV II, watermelon mosaic virus II; CYVV, clover yellow vein virus; BYMV, bean yellow mosaic virus; PeaMV, pea mosaic virus; TuMV, turnip mosaic virus. PC= personal communication.

Table II.1. Examples of Coat Protein Mediated Resistance: Expression of full length potyviral coat proteins in transgenic plants[a]

Host plant	CP expressed	Challenging virus	Resistance phenotype	Reference
N. tabacum cv. Xanthi nc	SMV	PVY (low)	R	Stark and Beachy, 1989; Thompson *et al.*, 1992
		PVY (high)	S	
		TEV(low)	R	
		TEV (high)	R	
		TVMV	S	
		TVBMV	S	
N. tabacum cv. Xanthi nc	PRV	TEV	R to HR	Ling *et al.*, 1991
		PVY	R	
		PeMV	R	
N.tabacum cv. Samsun	ZYMV	TEV	R	Fang and Grumet, 1993
Cucumis melo	ZYMV	ZYMV	CR	Fang and Grumet, 1993
S. tuberosum	PVY	PVY	S to HR	Lawson *et al.*, 1990; Kaniewski *et al.*, 1990; Farinelli *et al.*, 1992
N. tabacum	PVY	PVY	S to R	van der Vlugt *et al.*, 1992; Woloshuk *et al.*, 1992
N. benthamiana and N. clevelandii	PPV	PPV	R	Regner *et al.*, 1992
Carica papaya	PRV	PRV	S to CR	Fitch *et al.*, 1992
N. tabacum cv. Burley 49	TEV	TEV	R to HR	Lindbo and Dougherty, 1992a
		PVY	S	
		TVMV	S	
N. tabacum cv. Burley 21	TVMV	TVMV	S	Hunt, A. (PC)
		TEV	R	
		PVY	R	
N. benthamiana	WMV II or ZYMV	WMV II	HR	Namba *et al.*, 1992
		CYVV	R	
		BYMV	R	
		TEV	R	
		PeMV	R	
		PVY	R	
		PeaMV	R	
N. benthamiana	BYMV	BYMV	R	Hammond and Kamo, 1992
		PeMV	S	
		TuMV	S	

heterozygous (CP gene) plants and displayed a higher level of resistance to TEV or PVY challenge. They also noted that the degree of resistance could be reduced by challenging plants with higher inoculum levels and that older plants were more resistant than younger plants.

Thompson and colleagues recently re-examined the resistance of line 1052 by challenging the plants with high inoculum levels (infected plant sap) of TEV, PVY, tobacco vein mottling virus (TVMV), or tobacco vein banding mosaic virus (TVBMV) (Thompson *et al.*, 1992). Line 1052 again showed some resistance to TEV, but displayed little resistance to the other potyviruses tested. This work confirmed the earlier observation by Stark and Beachy that challenge inoculum dose could affect resistance.

The first report of virus-specific CPMR to a potyvirus involved transgenic potato plants transformed with the PVY CP gene alone, or co-introduced with the potato virus X (PVX) CP gene (Lawson *et al.*, 1990). The PVY gene construct inserted into the potato genome consisted of the entire PVY CP coding sequence and PVY 3' untranslated sequence (UTS). This construct resulted in the production of a PVY CP with minor amino acid changes at the N-terminus. Four transgenic lines were selected and tested for resistance against PVY and PVX. One line (303) showed apparent immunity to both PVY and PVX when challenged with these viruses. The three remaining lines showed varying degrees of resistance to PVY and PVX challenge. These transgenic potato lines also were shown to be resistant to aphid-vectored PVY inoculum. Field studies of these four transgenic lines have been performed (Kaniewski *et al.*, 1990). These studies resulted in observations similar to those obtained with plants grown under controlled conditions. Interestingly, as was observed in the study with SMV CP-expressing tobacco, there was no

correlation between accumulation of potyviral CP and the level of resistance observed; in fact, the most PVY-resistant line (line 303) accumulated little to no detectable PVY CP.

The Gonsalves research group (Cornell University, USA) has studied several examples of CPMR, investigating virus-specific and broad spectrum resistance (Ling *et al.*, 1991; Namba, *et al.*, 1992; Fitch *et al.*, 1992). A chimeric gene composed of cucumber mosaic virus- (CMV) and papaya ringspot potyvirus- (PRV) derived sequences was generated and inserted into the genome of *N. tabacum* cv. Havana 423 (Ling *et al.*, 1991). This gene produced a chimeric protein of 16 CMV amino acids (aa) fused to the amino terminus of the PRV CP. As tobacco is not a host for PRV, transgenic plant lines were challenged with TEV, PVY, or pepper mottle potyvirus (PeMV). Most of the eight tested transgenic plant lines displayed resistance to TEV, exhibited as a delay in symptom expression, attenuated symptoms, and lower virus titers as assayed by enzyme-linked immunosorbent assay (ELISA). The best protected line (I-26) did not display symptoms until 25-30 days after all controls were symptomatic. Plants of line I-26, however, tested positive for TEV by ELISA several days before symptoms appeared. Infected plants of line I-26 were observed to "outgrow" TEV-induced symptoms; that is, newly emerging leaves would demonstrate progressively fewer and fewer symptoms, until new growth appeared asymptomatic. Line I-26 also was demonstrated to be resistant to PeMV and PVY, showing a delay in symptoms of ca. 10 days for PeMV and ca. 5 days for PVY. As observed by Stark and Beachy (1989) and Lawson and co-workers (1991), plant lines accumulating the most CP were not the most resistant.

In an effort to compare the efficacy of virus-specific and broad spectrum CPMR, the Gonsalves group generated transgenic *N. benthamiana* plants expressing either the watermelon mosaic virus II (WMV II) CP- or zucchini yellow mosaic virus (ZYMV) CP-gene (Namba *et al.*, 1992). Transgenic plants were obtained that accumulated WMV II CP at levels of 0.1% to 0.7% of the total soluble protein. None of the ZYMV CP transgenic plants accumulated CP at a level greater than 0.1% total soluble protein. Three selected (R1 generation) lines of each of the two types of transgenic plants were challenged with WMV II. The best protected WMV II CP- and ZYMV CP-accumulating transgenic lines were analyzed further. These two transgenic plant lines were challenged with a battery of potyviruses including WMV II, bean yellow mosaic potyvirus (BYMV), PVY, pea mosaic potyvirus (PeaMV), clover yellow vein potyvirus (CYVV), PeMV, and TEV. The WMV II CP gene-expressing line showed delays in symptom expression with all potyviruses examined, but the extent of the delay and percentage of escapes (asymptomatic plants) varied with the particular virus and inoculum titer. In general, the plants were most resistant to challenge by WMV II; followed by the potyviruses BYMV, TEV, and PeMV; with the least resistance to PVY and PeaMV. In general, the WMV II CP transgenics displayed better resistance than transgenic plants accumulating ZYMV CP.

The ability of ZYMV CP to participate in CPMR also has been examined by Fang and Grumet (1993). Transgenic melon (*Cucumis melo* L. Hale's Best Jumbo) and tobacco plants (*N. tabacum* cv. Samsun) were generated which expressed the ZYMV CP gene sequence. The transgenic melons were protected against ZYMV challenge, while the transgenic tobacco only showed a slight delay in symptom development and reduction in virus titer (by ELISA analysis) when challenged with TEV or PVY.

Transgenic tobacco (*N. tabacum* cv. Burley 49) plants accumulating the TEV CP have been constructed and challenged with TEV, PVY, and TVMV (Lindbo and Dougherty, 1992a). In these studies, plants were challenged with high inoculum levels (1:10 dilutions of infected plant sap) of virus. Inoculation with TEV resulted in plants that developed symptoms at wild-type rates, but then "outgrew" the infection ca. 3-5 weeks post inoculation. These same plant lines were completely sensitive to infection with PVY and TVMV.

Transgenic tobacco (*N. tabacum* cv. Burley 21) expressing the TVMV CP gene have been generated and challenged with 1 μg/ ml of purified virus of TVMV, PVY, or TEV (A. Hunt, personal communication). Interestingly, such plants were more resistant to both TEV and PVY challenge than to TVMV challenge. This is one of the few cases where broad spectrum resistance appears more robust than virus-specific resistance.

Recently, two perennial plant species have been engineered to express potyvirus CP gene sequences. Transgenic papaya expressing PRV CP (Fitch *et al.*, 1992) and transgenic apricot expressing plum pox potyvirus (PPV) CP (Laimer da Câmara Machado *et al.*, 1992) have been generated. In the case of papaya, wild type sensitivities, intermediate, and high levels of resistance have been identified in separate transgenic plant lines. The resistance phenotype of transgenic apricot plants has not yet been reported.

CPMR with truncated or chimeric coat proteins

This section deals with transgenic plants which express altered forms of potyvirus CPs (Table II.2.). To date, only a limited number of plants expressing truncated or chimeric CPs have been described.

Table II.2. Examples of Coat Protein Mediated Resistance: Expression of truncated or chimeric potyvirus coat proteins in transgenic plants[a]

Host plant	CP form expressed	Challenging virus	Response to challenging virus	Reference
N. tabacum cv. Burley 49	TEV ΔN29	TEV	R	Lindbo and Dougherty, 1992a
		PVY	S	
		TVMV	S	
	TEV ΔC18	TEV	R	
		PVY	S	
		TVMV	S	
	TEV ΔN/C	TEV	R	
		PVY	S	
		TVMV	S	
N. tabacum cv. Samsun	ZYMV ΔN41	TEV	R	Fang and Grumet, 1993
		PVY	R	
Cucumis melo L. Hale's Best Junbo	ZYMV ΔN41	ZYMV	R	Fang and Grumet, 1993
N.benthamiana	BYMV/ZYMV	BYMV	R	Hammond and Kamo, 1992
		TuMV	S	
		PVY	S	
N.benthamiana	BYMV/PeMV	BYMV	R	Hammond and Kamo, 1992
		TuMV	S	
		PVY	R	

[a] Abbreviations used are as described for Table II.1.
Other abbreviations used are as follows: BYMV/ZYMV, BYMV-ZYMV chimeric coat protein(CP); BYMV/PeMV, BYMV-PeMV chimeric coat protein; TEV ΔN29, amino (N)-terminal 29 amino acids (aa) deleted from TEV CP; TEV ΔC18, carboxy(C)-terminal 20 aa deleted from TEV CP; TEV ΔN/C, N- and C-terminally truncated TEV CP; ZYMV ΔN41, ZYMV CP lacking the 41 N-terminal aa. Other abbreviations used are as described in Table II.1.

Potyvirus CPs contain an internal "core" sequence of amino acids (aa) which are highly conserved among the group (Shukla and Ward, 1989). The most variable regions of potyvirus CPs are adjacent to the amino- (N-) and carboxy- (C-) termini. In an effort to examine the ability of mutated forms of a potyvirus CP to confer resistance, we have generated transgenic plant lines which express one of several different forms of the TEV CP gene (Lindbo and Dougherty, 1992a), including truncations which flank the conserved "core" sequence of amino acids. Transgenic tobacco (*N. tabacum* cv. Burley 49) plants have been constructed which express the full length TEV CP (FL lines) or one of the following truncated forms of CP: (1) a CP lacking the N-terminal 29 aa (ΔN29 lines); (2), a CP form in which the C-terminal 20 aa were replaced by 7 non-TEV encoded amino acids (ΔC18 lines), or (3) a CP lacking both the N- and C- terminal sequences (ΔN/C lines).

We have examined 8 to 10 individual transformants of each line homozygous for the CP gene sequence (Lindbo and Dougherty, 1992a; unpublished results). Mechanical inoculation of these lines with high titers of TEV (1:10 dilution of TEV-infected plant sap) resulted in distinct symptoms for different lines. Transgenic plant lines that accumulated the ΔN29 form of TEV CP were similar to lines that express the FL version of the TEV CP gene in that they showed little or no delay in the initial appearance and severity of TEV-induced symptoms. However, TEV-infected plants from these lines eventually recovered (outgrew) and new growth was devoid of symptoms and virus. Transgenic plant lines expressing either ΔC18 or ΔN/C truncated forms of the TEV CP gene showed a significant delay in the appearance of TEV-induced symptoms. Additionally, in ΔC and ΔN/C lines, some plants never developed symptoms, and symptoms, when present, were attenuated. Symptomatic plants were systemically infected with TEV while asymptomatic tissue was

determined by ELISA and bioassay procedures to be free of virus. Protoplasts derived from all of these various TEV CP transgenic plant lines supported viral replication at levels similar to untransformed *N. tabacum* cv. Burley 49 protoplasts. This suggested that the various versions of the TEV CP were effective at interfering with virus movement inside the plant and not with virus replication *per se*.

Fang and Grummet (1993) have generated transgenic plants expressing a ZYMV CP lacking the amino terminal 41 amino acids. This truncated CP construct was not as effective as full length ZYMV CP in conferring resistance to ZYMV in transgenic melon, or to TEV or PVY in transgenic tobacco.

Hammond and Kamo have generated transgenic plants expressing chimeric CP genes composed of BYMV and PeMV (BYMV-PeMV) sequences, or BYMV and ZYMV sequences (BYMV-ZYMV) (Hammond and Kamo, 1992). Transgenic plants expressing the BYMV-ZYMV fusion protein had a resistance phenotype similar to transgenic plants which produced BYMV CP (*i. e.* BYMV resistant, TuMV and PVY sensitive). However, the BYMV-PeMV transgenic plant lines, while sensitive to TuMV, were resistant to both BYMV and to PVY. Upon DNA sequence analysis of the PeMV transgene component, the possibility was raised that the BYMV-PeMV chimera is actually a BYMV-PVY chimera. Resistance to PVY in these transgenic plants was possibly due to the "PeMV"-derived amino acids in the coat protein fusion, or the 3' UTS of the chimeric gene. This experiment raises the possibility of extending the spectrum of broad spectrum resistance through the construction of chimeric potyvirus CP genes.

Characteristics of CPMR

It is clear that virus CPs can be effective in conferring virus specific and broad spectrum resistance to potyviruses. Although the results are somewhat confusing, the following generalizations of CPMR can be made:

1. The resistance phenotype can be expressed as:
    a) a temporal delay in the development of symptoms.
    b) an attenuation of normal virus-induced symptoms.
    c) lower virus titer in infected transgenic tissue.
    d) the ability of infected plants to "outgrow" infection.
    e) a percentage of plants which escape infection.
2. Resistance phenotypes usually can be overcome by high levels of virus inoculum.
3. Resistance is more effective in older plants than in younger plants.
4. Virus-specific CPMR tends to be more effective than broad spectrum CPMR.
5. Truncated or chimeric forms of CPs can (in some cases) be used to increase the effectiveness and/or spectrum of CPMR.
6. There does not appear to be a correlation between CP accumulation and resistance.
7. Many lines which accumulate CP showed virus susceptibilities identical to untransformed parental tissue.

Transgenic plants accumulating nonstructural proteins

Although CP sequences have been the most thoroughly investigated for their ability to confer resistance, other potyviral genes have been examined as well. This section will discuss some of the results obtained with transgenic plants expressing nonstructural potyviral proteins (Table II.3.).

N. tabacum cv. Xanthi plants were generated by Berger and co-workers (Berger *et al.*, 1989) to express a polyprotein of the first three genes of TVMV [P1, helper component-proteinase (HC-PRO), and 42 K genes (see Fig. II.1)]. The P1 and HC-PRO proteins of potyviruses contain proteolytic activities which self-cleave at their carboxyl termini. As a result of these proteolytic activities, the transgenic plants accumulated fully processed HC-PRO which had biologically active helper component activity. These plants were not analyzed for steady state levels of the P1 or 42K proteins. Challenge inoculation of these transgenic plants with TVMV resulted in no protection, and symptom formation similar to untransformed controls (J. Shaw, personal communication).

Transgenic plants expressing the TVMV nuclear inclusion protein a (NIa) or cytoplasmic inclusion (CI) protein (see Fig II.1.) have been generated and preliminary screening for resistance to PVY, TEV, and TVMV performed (A. Hunt, personal communication). Transgenic *N. tabacum* cv. Burley 21 plants expressing the TVMV NIa protein or a fusion protein of NIa and the bacterial *glnH* (glutamine H) gene (NIa-glnH) were highly resistant to TVMV, but were susceptible to PVY and TEV. Only one transgenic plant line producing TVMV CI has been examined to date and has not displayed resistance to TVMV. *N. tabacum* cv. Xanthi nc expressing the TEV 49K (NIa), 58 K (NIb), or HC-Pro genes have been generated and accumulated the expected viral proteins (Carrington *et al.*, 1990; Restrepo-Hartwig and Carrington, 1992). Challenge

Table II.3. Examples of potyvirus nonstructural protein genes expressed in transgenic plants[a]

Host plant	Non-structural protein gene expressed	Challenging virus	Response to challenging virus	Reference
N. tabacum cv. Burley 21	TVMV NIa or TVMV NIa-glnH	TVMV	HR	A. Hunt (PC)
		TEV	S	
		PVY	S	
N. tabacum cv. Xanthi	TVMV HC-PRO	TVMV	S	J. Shaw (PC)
N. tabacum cv. Burley 21	TVMV CI	TVMV	S	A. Hunt (PC)
N. tabacum cv. Havana 425	TEV HC-PRO	TEV	S	J. Carrington (PC)
	TEV NIa	TEV	S	
	TEV NIb	TEV	S	

[a] Abbreviations used are as described for Tables II.1 and II.2. Other abbreviations used are as follows; NIa, nuclear inclusion protein a; NIa-glnH, NIa-glnH fusion protein; glnH, bacterial glutamine H gene; NIb, nuclear inclusion protein b; HC-PRO, helper component-proteinase, CI, cytoplasmic inclusion protein. See Fig II.1. for genomic location of these coding regions. (PC)= personal communication.

inoculation with TEV revealed these plants were susceptible to TEV at a level nearly identical to untransformed control plants (J.C. Carrington, unpublished observations).

Transgenic plants expressing potyviral RNA sequences

Untranslatable sense-stranded RNAs of the TEV coat protein gene sequence have been shown to confer TEV resistance (Table II.4.) in transgenic *N. tabacum* cv. Burley 49 plants (Lindbo and Dougherty, 1992a; 1992b). A single nucleotide insertion (frameshift mutation) and four point mutations were introduced in the TEV CP gene. These mutations introduced stop codons at the 5th, 6th, and 9th codons in the gene. The resulting mutated form of the TEV CP gene (called an RNA Control, or RC form) did not direct the synthesis of detectable levels of TEV CP *in planta*. Approximately 40% of the transgenic plant lines examined displayed a resistance phenotype which was absolute; no virus was detectable after challenge inoculation with TEV. The remaining RC lines, all expressing the untranslatable RNA, behaved as untransformed tobacco and displayed typical virus symptoms when inoculated. Protected transgenic lines withstood high inoculum doses of all isolates of TEV tested, but were susceptible to the potyviruses PVY and TVMV. Resistance was virus-specific, but not isolate specific. The resistance in RC transgenic plants was quite durable, as it was effective against mechanically- or aphid-transmitted TEV and also was effective in transgenic plants 2-3 weeks old (the youngest age tested). Protoplasts from resistant RC transgenic plants were transfected with TEV RNA. These cells did not support the production of detectable levels of TEV protein or infectious particles. However, as observed with coat protein expressing transgenic lines, a correlation between steady state RNA levels and protection could not be established.

Table II.4. RNA-mediated resistance to Potyviruses[a]

Host plant	RNA expressed	Challenge virus	Response to challenge virus	Reference
N. tabacum cv. Burley 49	TEV-CP AS RNA	TEV PVY TVMV	S or R S S	Lindbo and Dougherty, 1992a; 1992b
N. tabacum cv. Burley 49	TEV-CP RNA Control (RC)	TEV PVY TVMV	S or CR S S	Lindbo and Dougherty, 1992a; 1992b
N. tabacum cv. SR1	PVY-CP RNA (-ATG)	PVY	S or R	van der Vlugt *et al..*, 1992
N. benthamiana	BYMV-CP AS RNA	BYMV TEV TuMV	S, R or CR S S	Hammond and Kamo, 1992
Cucumis melo L. 'Hale's Best Jumbo'	ZYMV-CP AS RNA	ZYMV	R	Fang and Grumet, 1993
N. tabacum cv. Samsun	ZYMV-CP AS RNA	TEV PVY	R R	Fang and Grumet, 1993

[a]Abbreviations used are as described for Table II.1. Other abbreviations are as follows: AS, antisense; RC, RNA Control (untranslatable sense RNA); (-ATG), CP gene lacking an ATG start codon.

Transgenic tobacco (*N. tabacum* cv. SR1) plants expressing the PVY CP gene lacking an AUG start codon (PVY CP $^{-ATG}$) have also been generated. Such transgenic plant lines express a sense strand PVY CP RNA but no CP. Approximately 20% of the transgenic plant lines tested were reported to be resistant to PVY (van der Vlugt *et al.*, 1992).

Selected transgenic plants accumulating potyvirus complimentary, or antisense (AS), RNA sequences have been shown to exhibit a virus-resistant phenotype. Antisense RNA-mediated resistance has been reported for the potyviruses ZYMV, BYMV, and TEV (Fang and Grumet, 1993; Lindbo and Dougherty, 1992a; Hammond and Kamo, 1992) (Table II.4.). In some cases the transgenic constructs have included viral-derived 3' UTS (Fang and Grumet, 1993; Hammond and Kamo, 1992), though the effect of expressing this portion of the potyvirus genome is not known. When challenged with virus, transgenic plants expressing AS sequences responded to virus infection by displaying phenotypes which ranged from wild-type sensitivities to apparent complete resistance. Only in plants expressing ZYMV CP AS RNA, was a low level of broad spectrum resistance detected; BYMV and TEV CP AS RNA plants displayed only virus specific resistance. In these studies there was no consistent correlation between steady state levels of antisense transcripts and resistance. These observations are reminiscent of the results observed for transgenic plant lines expressing potyviral CP sense mRNA and CP.

Summary and Discussion

The disparate results obtained for PDR with potyviruses make it difficult to hypothesize a single unifying mechanism for resistance. Interpretation of results is further complicated by the wide variety of systems and genetic constructs used. In general, experiments designed to investigate the mechanism(s) of resistance are limited. Below, I detail some working models which may explain some results summarized in this chapter.

In its simplest form, virus resistance could be due to the transgene product directly interfering with one (or more) of several viral processes such as replication, assembly, or movement. In our studies (Lindbo and Dougherty, 1992a) with transgenic plants expressing a FL TEV CP, TEV-challenged plants initially developed symptoms and virus titers similar to wild-type plants. Similarly, protoplasts from these transgenic plants appeared to support virus replication at wild-type levels. However, these transgenic plants consistently outgrew infection and young emerging leaf tissue was asymptomatic and devoid of virus. Untransformed control plants never were observed to outgrow TEV infection. Collectively, these results suggest virus movement might have been impaired, especially as the infected plant grew older. However, some recent preliminary studies could require modification of this simplistic model. In a study in which the upper virus-free leaves of a "recovered" FL transgenic plant were challenged with TEV, a systemic or localized infection could not be established (WGD, unpublished observations). Thus it appears as though a "virus resistant" state had been achieved in these leaves. This virus resistant state may be host-encoded and induced by the interaction of the transgene, viral, and host genomes. A similar phenomenon occurs in transgenic plants which express ΔN29 truncated forms of the TEV CP.

Perhaps most perplexing in the phenomenon of CPMR is the lack of correlation between CP gene expression level and resistance. In many of the examples cited in this review, transgenic plants expressing potyvirus CPs showed little or no resistance in response to challenge with virus. All gene expression estimates, however, have been made at a very gross level (i.e. expression levels of a piece of tissue have been extrapolated to represent expression on a per cell or whole plant basis). This may not be entirely appropriate. Due to "position effects," different gene expression levels and patterns (Barnes, 1990) (cell-specific expression patterns) can exist in different transgenic plants containing the same transgene. It may be that all cells (or only a specific subset of cells) of a plant or tissue need to express a certain basal level of transgene product to confer resistance. Since transgene expression analysis has not yet been performed on a cellular level, it is currently impossible to determine if this is the case.

Therefore, we are left with a variety of CPMR studies in which no clear mechanistic theme can be definitively applied to all examples. It is conceivable that no one single mechanism dominates or exists. Rather, multiple mechanisms may be in effect and operate with different efficiencies in different transgenic plants and different host-pathogen systems. Since potyvirus genomes are small (compared to other biotic pathogens), it is reasonable to expect that their proteins are often multifunctional. Therefore, expression of one virus gene product could theoretically alter multiple viral and/or host processes. Much work remains to be done on elucidating the mechanism of CPMR toward potyviruses. A central question to be initially addressed is whether the coat protein is directly or indirectly responsible for the resistance phenotype in transgenic plants.

Most transgenic plants expressing a potyvirus non-structural gene were not virus resistant. The exception to this was transgenic tobacco plants expressing the TVMV NIa gene. These lines were resistant to TVMV, while similar plants expressing TEV NIa genes were not resistant to TEV. The TEV NIa protein is known to be directed to the nucleus (Restrepo-Hartwig and Carrington, 1992) when expressed *in planta*, whereas it is not known if the TVMV NIa protein has this characteristic. The nuclear localization of the TEV NIa protein may prohibit it from interfering with cytoplasmically localized viral functions. Although results with transgenic plants expressing potyvirus non-structural proteins have been, in most cases, disappointing, these observations do not necessarily infer that these proteins will be unable to confer resistance. These negative results may instead be a result of small sample size and the preliminary nature of these studies. Alternatively, mutated forms of these genes could be effective in conferring viral resistance.

A remarkably high degree of (virus specific) resistance has been obtained with plants expressing untranslatable sense or antisense CP RNAs. It has been proposed that these untranslatable RNAs interfere with virus replication, perhaps by hybridizing to plus or minus sense virus genomes. In transgenic plants expressing an untranslatable plus-sense RNA (RC), resistance was dramatic. In the TEV system, the resistance was TEV-specific and independent of plant age or virus inoculum levels. No TEV replication could be detected in resistant transgenic plants or protoplasts. Complicating this system, however, was the observation that not all plant lines expressing an RC CP RNA sequence were resistant. Approximately 40% of the independent selections of the various RC-producing transgenic lines display resistance. I have proposed that the RC molecule inhibits replication by hybridizing to minus sense TEV RNA genomes. I have also suggested that the failure of some

lines to display the resistant phenotype was attributable to tissue specific expression patterns. However, in light of preliminary data regarding the apparent virus resistant state in "recovering" CP-producing transgenic plants (see discussion above), I believe it prudent to ask if the untranslatable RNA actually is the molecule in the host cell which arrests virus replication. For example, could the RC or AS RNAs induce a host-mediated virus resistant state similar to what may be happening in CP-expressing plants that "outgrow" infection? Perhaps, RC or AS gene constructs can interfere with the expression of host or virus genes, by a phenomenon similar to "co-suppression" reported for some transgenic plants (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; de Carvalho *et al.*, 1992). A better understanding of these phenomena could lead to important improvements in the generation (and understanding) of virus resistance in plants.

In addition to providing effective resistance, RNA-mediated resistance has a number of advantages over protein-mediated resistance strategies. Concerns have been raised as to the possibility of "new" viable viruses being formed through transencapsidation of viral genomes with CPs expressed from transgenic plants, or by RNA-recombination between a virus genome and a transgene mRNA (de Zoeten, 1991). It has been recently demonstrated that transencapsidation can occur in transgenic plants (Farinelli *et al.*, 1992) Both of these concerns could be alleviated by expressing dysfunctional viral genes (such as untranslatable RNA sequences) in transgenic plants. Additionally, regulatory approval of plants expressing a mutated RNA may be less problematic than a plant expressing both a viral RNA and protein. Finally, the RNA-mediated resistance rendered the plant highly resistant to virus replication and, as a result, may be very difficult for a virus to overcome. I suggest the virus will not have the opportunity to replicate and adapt to the

RC-producing cells. Over time this may be a limitation of CPMR since it does allow a reduced level of virus replication.

In conclusion, it is encouraging that, in addition to the well established phenomenon of CPMR, alternative effective resistance strategies are appearing. These strategies include the expression of mutated (dysfunctional?) CPs, chimeric CPs, non-structural proteins, and RNA (sense or antisense) mediated resistance. Further studies performed on each of these different strategies may lead to our understanding of the mechanism(s) involved in virus resistance allowing for a more rational approach in designing virus resistant plants.

CHAPTER III

PATHOGEN DERIVED RESISTANCE TO A POTYVIRUS: IMMUNE AND RESISTANT PHENOTYPES IN TRANSGENIC TOBACCO EXPRESSING ALTERED FORMS OF A POTYVIRUS COAT PROTEIN NUCLEOTIDE SEQUENCE

Abstract

Transgenic *Nicotiana tabacum* cv. Burley 49 plants containing one of six different forms of the Tobacco Etch Virus (TEV) coat protein (CP) nucleotide sequence have been generated. In whole plant studies, R1 and R2 progeny were inoculated mechanically with TEV and the appearance and severity of symptoms recorded. Symptom phenotype was altered, ranging from near wild-type susceptibility to apparent immunity. Protoplasts derived from wild-type and transgenic Burley 49 plant lines were transfected with TEV RNA. Protoplasts from transgenic plants expressing full-length or truncated forms of TEV CP supported virus replication. Protoplasts from certain transgenic plants, producing plus- or minus-sense CP transcripts but no CP, did not support virus replication at wild-type levels. A model is proposed to account for these observations.

Introduction

The theory of pathogen derived resistance (Sanford and Johnston, 1985) predicts that a "normal" host-pathogen relationship can be disrupted if the host organism expresses certain pathogen derived genes. It has been proposed that host organisms expressing pathogen gene products in excess amounts, at the inappropriate developmental stage, or in a dysfunctional form may disrupt the normal replicative cycle of the pathogen and result in an attenuated or aborted infection of the host.

It has been demonstrated that transgenic plants expressing a plant virus coat protein can be resistant to infection by the homologous virus. This type of pathogen derived resistance has come to be known as coat protein-mediated resistance and has been demonstrated for the tobamo-, potex-, cucumo-, tobra-, carla-, poty- and alfalfa mosaic virus groups (for review see Beachy *et al.*, 1990) and more recently for the luteovirus group (Kawchuk *et al.*, 1990, 1991). I have undertaken experiments to investigate the efficacy and mechanism of this form of pathogen derived resistance for the potyvirus group.

The potato virus Y, or *Potyvirus* genus, contains a large number of plant viral pathogens which collectively can infect most crop species and compromise crop yield and/or quality (Hollings and Brunt, 1981; Matthews, 1982; Francki *et al.*, 1985). Potyviruses have a single-stranded, "plus sense" RNA (of about 10,000 nucleotides) which has a viral-encoded protein linked to the 5' end and a 3' polyadenylate region. A single open reading frame (ORF) codes for a 351,000 dalton (351 kDa) polyprotein which is proteolytically processed into mature viral gene products (Allison *et al.*, 1985b, 1986b; Dougherty and Carrington, 1988). The RNA is encapsidated by

approximately 2000 copies of a coat protein monomer to form a virion (Hollings and Brunt, 1981). The capsid protein is encoded by the sequence present at the 3' end of the large ORF (Allison *et al.*, 1985b)

I have generated a series of transgenic plants expressing either full-length or truncated forms of the coat protein (CP) of the potyvirus tobacco etch virus (TEV). Additionally, transgenic plants were generated which expressed either an antisense (AS) form of the CP RNA sequence or a "sense" stranded CP RNA molecule (RC) with a frameshift mutation, rendering it untranslatable. R1 and R2 progeny plants were screened by Western and/or Northern blot analysis for expression of the transgene. Expressing plant lines were inoculated mechanically with TEV and symptoms recorded. Truncated forms of the TEV CP tended to confer greater protection than the full-length CP. Select plant lines expressing either AS or RC transcripts were highly resistant. In protoplast transfection studies, all lines which produced CP supported viral replication, while those lines which expressed high levels of AS or RC RNA did not support replication at wild-type levels. These results suggest that the resistant phenotypes observed function *via* at least two different mechanisms in these transgenic plant lines.

Materials and Methods

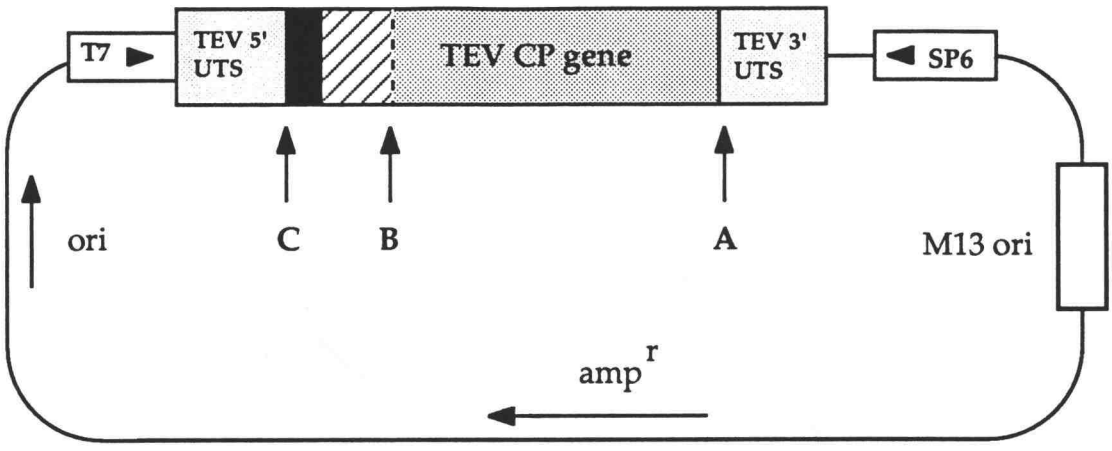
All restriction enzymes were purchased from New England Biolabs, unless specified, and used according to manufacturers instructions. Plasmids were maintained in *E. coli* strains HB101 or TG1. Radioactive isotopes were purchased from New England Nuclear. T7 and SP6 RNA polymerases were purchased from Bethesda Research Laboratories. Rabbit reticulocyte lysate was purchased from Green Hectares, Oregon, WI. Plasmid pCGN 2113 was a kind gift from Calgene (Davis, California). *Agrobacterium tumefaciens* strain A136/pCIB 542, pRK2013, pCIB 710, and pCIB 200 were kind gifts of Ciba Geigy Corporation (Research Triangle Park, North Carolina).

Mutagenesis of pTL 37/8595

Plasmid pTL 37/8595 (Carrington and Dougherty, 1987; Carrington *et al.*, 1987) contains a cDNA copy of the genomic sequence of the highly aphid transmissible (HAT) isolate of TEV corresponding to nucleotides (nt) 1-200 and nt 8,462-9,495. The first and last codons of the CP coding region in the TEV genome are nt 8,518-8,520 (Ser) and 9,307-9,309 (opal), respectively. (For numbering of TEV nucleotides, see Allison *et al.*, 1986). pTL 37/8595 was subjected to *in vitro* site-directed mutagenesis using the method developed by Taylor *et al.* (1985 a,b). In all cases, nucleotide changes were confirmed by dideoxy-nucleotide sequencing (Sanger *et al.*, 1977).

TEV nt 9,312-9,317 were first mutated (Fig. III.1) to generate a *Bam*HI restriction site. TEV nt 8,516-8,521 were then altered to generate an *Nco*I site, changing the first codon of the TEV CP coding region from AGT (Ser), to ATG (Met). A single oligonucleotide was then used to mutate TEV nt 133-

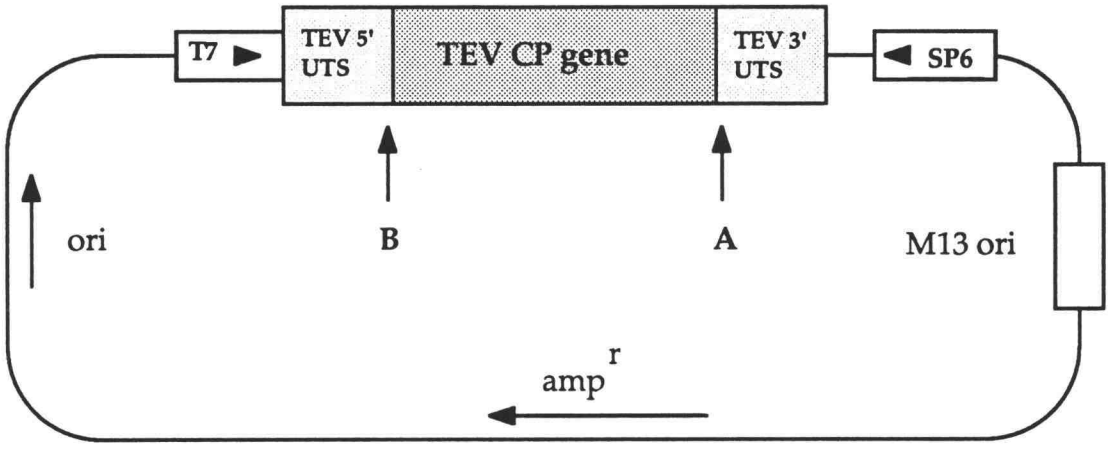
Fig. III.1. Schematic representation of pTC: FL and its construction. Restriction endonuclease sites were introduced into pTL 37/8595 at positions A, B and C in diagram. Following these nucleotide changes the mutated pTL 37/8595 was digested with the restriction enzyme *Nco*I, the DNA fragment delineated by the restriction enzyme sites at B and C was removed, and the plasmid religated to generate pTC: FL. pTC:FL contains the tobacco etch virus (TEV) coat protein nucleotide sequence flanked by *Bam*HI restriction sites and the TEV 5' and 3' untranslated sequences (UTS). T7 and SP6 promoters are also shown. Abbreviations used in this diagram are as follows: T7, T7 RNA polymerase promoter sequence; SP6, SP6 RNA polymerase promoter sequence; ori, origin of replication; M13 ori, bacteriophage M13 single-stranded origin of replication; amp^r, β-lactamase gene. Lightly stippled areas are TEV 5' and 3' untranslated sequences; solid black area, TEV genome cDNA nucleotides 144 to 200; striped area, a portion of the TEV NIb gene (TEV nt 8,462-8,517); heavily stippled areas, cDNA of TEV CP nucleotide sequence (TEV nt 8,518-9,309).



pTL 37/8595

1. Generate BamHI site at **A** (nt 9,312-9,317)
2. Generate NcoI site at **B** (nt 8,516-8,521)
3. Generate BamHI site (nt 133-138)
   NcoI site (nt 143-148 and
   deoxyadenylate residue at **C**.

Digest with NcoI
remove TEV nucleotides 143-200/8,462-8,516
(flanked by sites B and C and religate.)



pTC :FL

Figure III.1

138 to a *Bam*HI restriction site, nt 143-148 to an *Nco*I restriction site and nt 142 to a deoxyadenylate residue. These mutations generated an *Nco*I site centered on the first codon of the TEV ORF and in a good translational start context as described by Kozak (1984). Digestion of the resulting plasmid with the restriction enzyme *Nco*I, removing TEV nt 143-200/8,462-8,516, and religation generated plasmid pTC:FL. pTC:FL contained the TEV CP gene flanked by *Bam*HI restriction sites and TEV 5' and 3' untranslated sequences (see Fig III.1).

Plasmid pTC:ΔN29 was constructed as follows: TEV nt 8,600-8,605 in pTC:FL were mutated to an *Nco*I restriction site. Digestion of the resulting plasmid with the restriction enzyme *Nco*I, removal of TEV nt 8,517-8,601, and religation generated pTC:ΔN29, a plasmid lacking the nucleotides which coded for the N-terminal 29 amino acids of the TEV CP gene.

pTC:FL and pTC:ΔN29 were modified further to generate plasmids pTC:ΔC18 and pTC:ΔN/C, respectively. Originally these two plasmids were mutated to create a premature stop codon at TEV nt 9,253-9,255, such that the 18 carboxy-terminal codons would not be translated. However, subsequent nucleotide sequence analysis of the mutagenized region revealed that a single nucleotide deletion (TEV nt 9,249) and three point mutations (at TEV nt 9,250-9,252) had also occurred. The resulting frameshift, 20 codons upstream of the wild type TEV CP stop codon, negated the introduced TAG stop codon at TEV nt 9,253-9,255 and created an in-frame TGA stop codon eight codons downstream of the frameshift. Translation of these genes resulted in a truncated TEV CP with the addition of seven non-TEV amino acids (EPRQRTM) after TEV CP amino acid number 243.

Plasmid pTC: RC (RNA control) was generated by insertion of a single deoxythymidylate residue after TEV nt 8,529, and point mutations of TEV nt 8,522 (G-C), 8,534 (C-A), 8,542 (G-A),and 8,543(A-G) to create a frameshift mutation immediately followed by three stop codons. An *Nhe*I restriction site was simultaneously generated, for screening purposes, at nt 8,539-8,544.

All plasmids described above were linearized with *Hind*III, transcribed with T7 RNA polymerase (Melton *et al.* 1984), and translated in a rabbit reticulocyte lysate containing ^{35}S methionine (Dougherty and Hiebert, 1980a). Radiolabeled translation products were analyzed by electrophoretic separation on a 12.5% acrylamide gel containing SDS (Laemmli, 1970) and detected by autoradiography. Transcripts of plasmid pTC:RC produced no detectable protein products while transcripts from all other plasmids produced proteins of the expected sizes (data not shown).

The various forms of the CP nucleotide sequence were then inserted as *Bam*HI cassettes into the plant expression vector pPEV (See below and Fig. III.2). The full length TEV CP ORF was inserted in the reverse orientation to make the antisense (AS) construct.

Construction of pPEV

The vector pPEV is part of a binary vector system for *Agrobacterium tumefaciens* mediated plant cell transformation. Plasmid pPEV was constructed from the plasmids pCGN 2113 (Calgene), pCIB 710 and pCIB 200 (Ciba Geigy Corp.). pCGN 2113 contains the "enhanced" Cauliflower Mosaic Virus (CaMV) 35S promoter (CaMV sequences -941 to -90/-363 to +2, relative to the transcription start site) in a pUC derived plasmid backbone. pCIB 710 has been described (Rothstein *et al.*, 1987) and pCIB 200 is a derivative of the

Fig III.2. Forms of the TEV coat protein gene inserted into *Nicotiana tabacum* cv. Burley 49. All constructs contained the enhanced CaMV 35S (Enh 35S) promoter, CaMV 35S 5' untranslated sequence (UTS) of 50 bp and the CaMV 35S 3' UTS/polyadenylation site of 110 bp. The nomenclature used to describe the transgenic plant lines is presented along with the gene products produced in those plant lines (far right column). Abbreviations used are as follows: 35S, transgenic plants containing the CaMV 35S promoter and 5' and 3' UTS only; FL, ΔN29, ΔC18, ΔN/C, transgenic plants containing the transgene coding for full length TEV CP, TEV CP lacking the amino-terminal 29 amino acids (aa), TEV CP lacking the carboxy-terminal 20 aa, or TEV CP lacking both the amino-terminal 29aa and carboxy-terminal 20 aa, respectively. AS and RC transgenic plants contain the transgene expressed as an antisense form of the TEV CP gene, or an untranslated sense form of the TEV CP gene, respectively. Arrowheads denote the presence of in-frame stop codons. The various forms of the TEV CP nucleotide sequence are shaded.

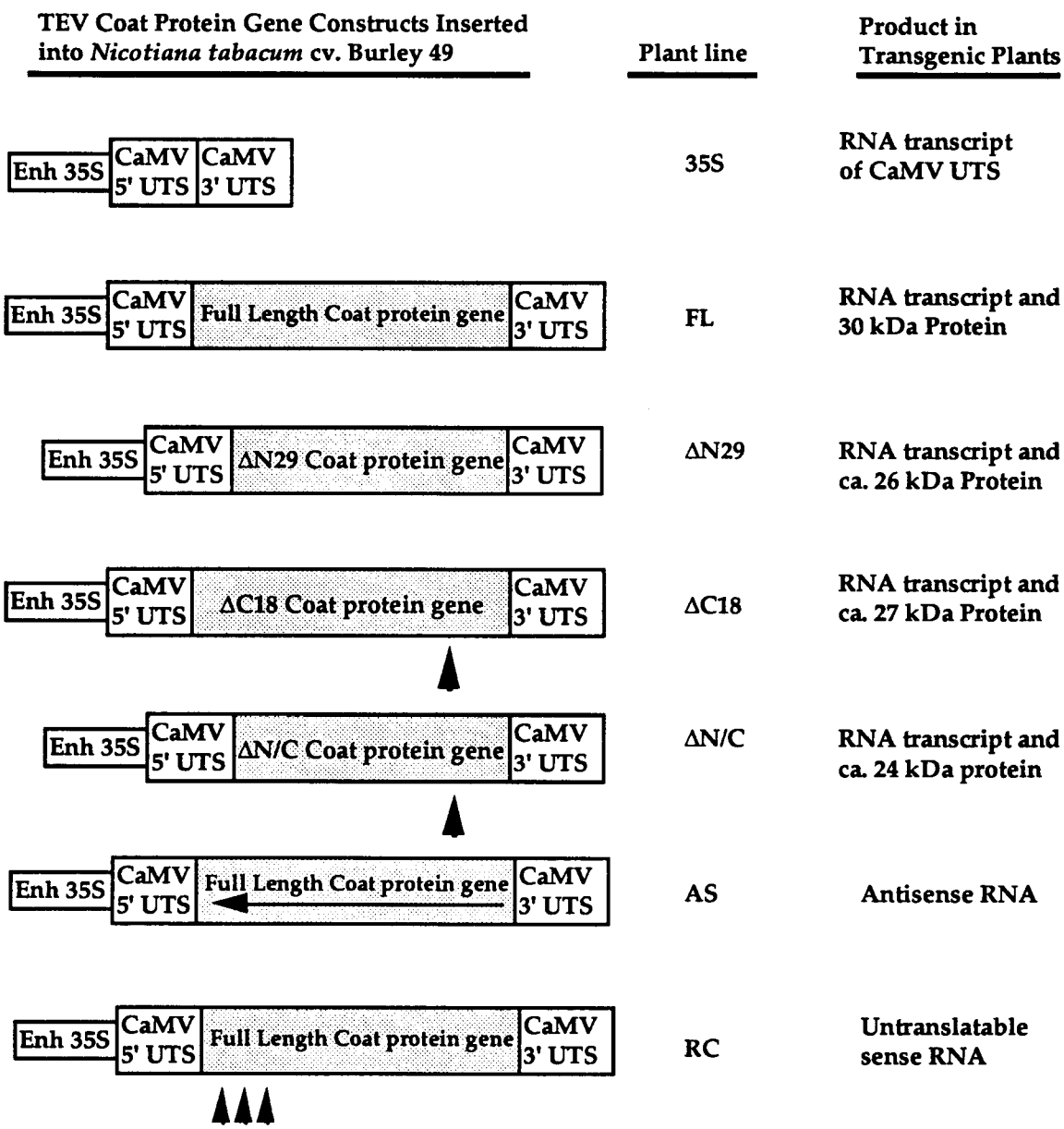

TEV Coat Protein Gene Constructs Inserted into Nicotiana tabacum cv. Burley 49
Plant line
Product in Transgenic Plants
Enh 35S
CaMV 5' UTS
CaMV 3' UTS
35S
RNA transcript of CaMV UTS
Full Length Coat protein gene
FL
RNA transcript and 30 kDa Protein
ΔN29 Coat protein gene
ΔN29
RNA transcript and ca. 26 kDa Protein
ΔC18 Coat protein gene
ΔC18
RNA transcript and ca. 27 kDa Protein
ΔN/C Coat protein gene
ΔN/C
RNA transcript and ca. 24 kDa protein
AS
Antisense RNA
RC
Untranslatable sense RNA

Figure III.2.

wide host range plasmid pTJS 75 (Schmidhauser and Helinski, 1985) which contains left and right *A. tumefaciens* T37 DNA borders, the plant selectable NOS/NPT II chimeric gene from the plasmid Bin 6 (Bevan, 1984) and part of a pUC polylinker. The small *Eco*RI-*Eco*RV DNA fragment of pCIB 710 (Rothstein *et al.*, 1987) was ligated into *Eco*RI-*Eco*RV digested pCGN 2113. This regenerated the enhanced CaMV 35S promoter (Kay *et al.*, 1987) of pCGN 2113 and introduced the CaMV 35S 5' and 3' untranslated sequences into pCGN 2113. The CaMV 35S promoter-terminator cassette of the resulting plasmid was isolated as an *Eco*RI-*Xba*I DNA fragment and ligated into *Eco*RI-*Xba*I digested pCIB 200 to generate pPEV. TEV CP ORFs were cloned as *Bam*HI cassettes into *Bam*HI digested pPEV and orientation of inserts confirmed by digestion with appropriate restriction endonucleases.

Plant Transformation

pPEV plasmids containing TEV CP ORFs were mobilized from *E. coli* HB101 into *A. tumefaciens* A136 containing plasmid pCIB 542 (Ciba Geigy), using the helper plasmid pRK 2013 in *E. coli* HB101 and the tri-parental mating system of Ditta *et al.*(1980). Plasmid pCIB 542 supplied *vir* functions necessary for T-DNA transfer.

Leaf discs of *Nicotiana tabacum* cv. Burley 49 were transformed and whole plants regenerated according to Horsch *et al.* (1985). Transformed tissue was selected by culturing callus on MS plates (Murashige and Skoog, 1962) containing 1μg/ml 6-benzylaminopurine (Sigma Corp.), 0.1 μg/ml α-naphthaleneacetic acid (Sigma Corp.), 500 μg/ml carbenicillin and 100 μg/ml kanamycin sulfate (Sigma Corp.). Shoots were rooted on MS plates

containing 500 μg/ml carbenicillin and 100 μg/ml kanamycin sulfate and plantlets were transplanted into soil and transferred directly into the greenhouse approximately 2-3 weeks after rooting.

R0, R1 and R2 plants were screened by Western and/or Northern blot analyses. R2 seeds (ca. 100 seeds per R2 plant) are screened for Kan^r phenotype by surface sterilizing seed in 10% bleach for 5 min., washing twice in sterile water and germinating on MS plates containing 100 μg/ml kanamycin sulfate. R2 seed lines which were 100 % kanamycin resistant were screened by Western blot analysis for expression of TEV coat protein. Those transgenic plant lines generated and their nomenclature are presented in Fig. III.2.

Western Blot Analysis

Tissue samples of transgenic plants were ground in 10 volumes of 2X Laemmli (Tris-glycine) running buffer (Laemmli, 1970) and clarified by centrifugation in a microcentrifuge for 10 min at 10,000xg. Protein concentration was estimated by the dye binding procedure of Bradford (1976) using BSA as a standard. Protein samples (50 μg total protein) were separated on a 12.5% polyacrylamide gel containing SDS and subjected to the immunoblot transfer procedures described by Towbin *et al.* (1979). Anti-TEV coat protein polyclonal primary antibodies, alkaline phosphatase conjugated secondary antibodies and the chromogenic substrates NBT (para-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indoyl phosphate para-toluidine salt) were used to detect bound antigen.

Northern Blot Analysis

Total nucleic acids were isolated from tissue and RNA precipitated with LiCl as described by Verwoerd *et al.* (1989). RNAs were electrophoretically separated on 1.2 % agarose gels containing 6% (v/v) formaldehyde and transferred to nitrocellulose. Prehybridization and hybridization conditions were as described in Sambrook *et al.* (1989). Strand specific riboprobes were generated from SP6 or T7 DNA dependent RNA polymerase transcription reactions of pTL 37/8595 linearized with the restriction enzymes *Asp*718 (Boehringer Mannheim) or *Hind*III, respectively. Probes were labelled with α-^{32}P-CTP ribonucleotide and suggested procedures (Promega).

Inoculation of transgenic plants

Eight week old (ca. 15 cm tall) R1 and R2 plants were inoculated with either purified virus preparations or infected plant sap. Inoculum was applied with sterile, premoistened cotton swabs. Infected plant sap inoculum was prepared by grinding TEV-infected *N. tabacum* Burley 21 leaf tissue (2 weeks post-inoculation) in carborundum and 50 mM sodium phosphate buffer (pH 7.8) at a ratio of 1gm:0.2gm:10mls, respectively, and filtering the homogenate through cheesecloth. TEV virions were purified as described by Dougherty and Hiebert (1980b). One leaf per plant was dusted lightly with carborundum (320 grit) and inoculated at two interveinal locations with 50 µl (total) of inoculum. Inoculated plants were examined daily and the appearance and severity of systemic symptoms recorded. Symptoms on any leaf above the inoculated leaf were considered to be systemic. Ten R2 expressing plants of each of the CP expressing lines were inoculated with infected plant sap and 20 R1 plants of lines AS #3 and RC #5

were inoculated with 50 µl of a 5µg/ml solution of purified TEV. Identical results were obtained when AS #3 and RC #5 R1 plants were inoculated with TEV-infected plant sap, as described above.

Preparation, inoculation and analysis or protoplasts

Protoplasts were prepared from transgenic plants and electroporated according to the procedure of Luciano *et al.* (1987). Protoplasts (1×10^{6}) were resuspended in 450 µl electroporation buffer (330 mM mannitol, 1 mM potassium phosphate, pH 7.0, 150 mM KCl) and electroporated using a BTX Transfector 300 (San Diego, USA) (950 microFarads, 130 volt pulse amplitude, 3.5 mm electrode gap) in the presence or absence of 6 µg of purified TEV RNA. After electroporation, protoplasts were incubated for 96 hours in incubation medium as described in Luciano *et al.* (1987). Protoplasts were extracted in 2X Laemmli (Tris-glycine) running buffer and 5×10^{4} extracted protoplasts were then subjected to Western blot analysis as described above. Protoplast viability was measured by dye exclusion as described in Luciano *et al.* (1987). All electroporated protoplast samples had equivalent viability counts.

Results

Analysis of transgenic plants

I first sought to determine if the resulting transgenic Burley 49 tobacco plants were producing the expected RNA and protein products. Total RNA samples isolated from the various transgenic lines were analyzed in Northern blot hybridization studies. An RNA transcript of approximately 1,000 nt was expected with all transgenic plant lines. Such a TEV CP transcript was detected in CP expressing plant lines by using a "minus" sense-stranded riboprobe containing the TEV CP sequence (Fig. III.3, A and B, lanes 1-5). A similar transcript was detected in AS plants by using a "plus" sense-stranded riboprobe containing the TEV CP sequence (Fig. III.3B, lane 9). However, the transcript in the RC line (Fig. III.3B, lane 3), while detected with a "minus" sense riboprobe, may have migrated as a slightly larger (ca. 1100-1200 nt) RNA species, possibly due to termination at an alternately selected site and/or a longer poly-A tail on the transcript. Differing levels of CP transcript accumulation were observed among different transgenic plant lines (Fig. III.3.)

Transgenic plant lines expressing either full-length or truncated TEV CP were identified by Western blot analysis using polyclonal antisera to TEV CP. The various CP products produced in plants were stable and accumulated to different levels in individual transgenic plant lines. It was estimated by Western blot analysis that between 0.01% to 0.001 % of total extracted protein was TEV CP. Full-length and the three truncated forms of TEV CP could be readily distinguished in this analysis. Additionally, the antisera cross-reacted with two high molecular weight proteins present in

Fig. III.3. Northern blot analysis of total RNA extracted from transgenic plants. Samples (10 μg) of total RNA from R2 (coat protein [CP] lines) or R1 (AS #3 and RC #5) transgenic plants were separated on a 1.2% agarose gel containing 6% formaldehyde. RNAs were transferred to nitrocellulose membranes and hybridized with either minus-sense TEV CP riboprobe (**A** and lanes 1-5 of **B**) or with plus-sense TEV CP riboprobe (**B**, lanes 6-10). After washing under stringent conditions, filters were exposed to Kodak X-Omat film at -70° C. Plus or minus sense transcripts of pTL 37/8595 were used as markers. RNA present in each lane is as follows: **A.** Total RNA from 35S #4 leaf tissue with marker transcripts derived from T7 polymerase transcription of pTL 37/8595 added (Lane 1), total RNA samples from leaf tissue of R2 transgenic plants from lines 35S #4 (lane 2), FL #3 (lane 3), FL #24 (lane 4), ΔC18 #7 (lane 5), ΔC18 #15 (lane 6), ΔN29 #1(lane 7), ΔN29 #2 (lane 8), ΔN29 #8 (lane 9), ΔN/C #69 (lane 10), and ΔN/C #61 (lane 11). Marker transcript sizes are presented on the left side of the figures. **B.** Total RNA from leaf tissue of the following transgenic plant lines: 35S #4 with sense strand transcripts derived from pTL 37/8595 added (lanes 1 and 6), 35S #4 (lanes 2 and 7), RC #5 (lanes 3 and 8), AS #3 (lanes 4 and 9), 35S #4 with antisense strand transcripts (from SP6 transcription of pTL 37/8595) added (lanes 5 and 10). The sizes of the marker RNAs are presented on the left and right sides of the figure.

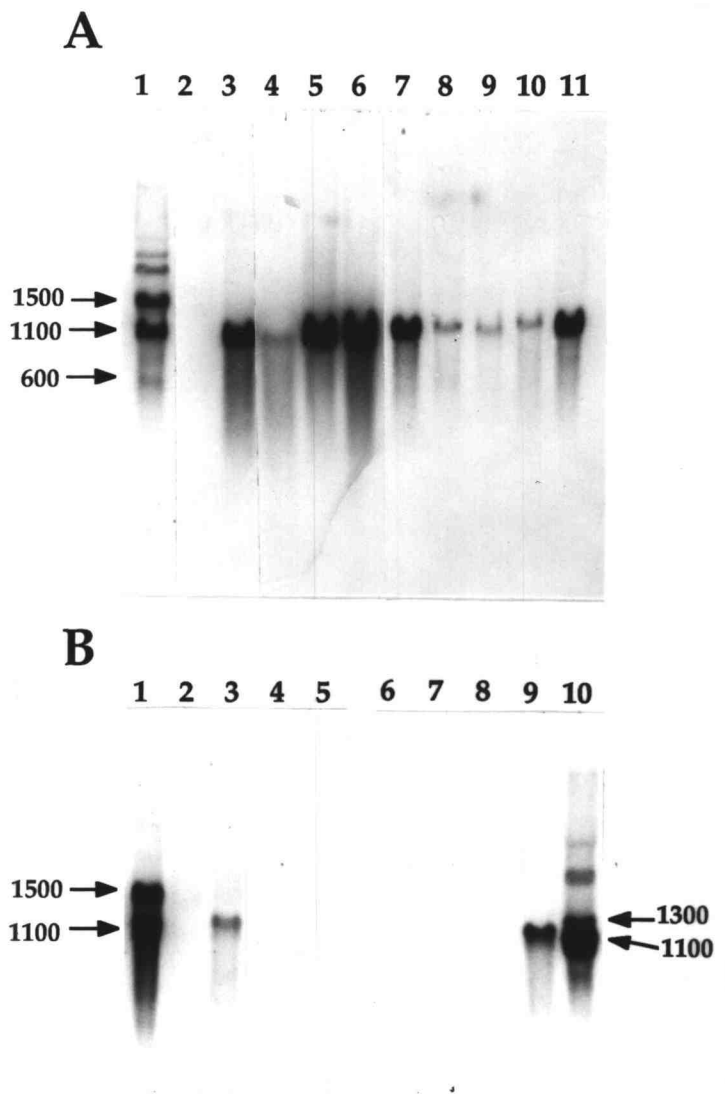

A
1 2 3 4 5 6 7 8 9 10 11
1500
1100
600
B
1 2 3 4 5 6 7 8 9 10
1500
1100
1300
1100

Figure III.3.

Burley 49. No CP was detected in extracts of the AS or RC transgenic plant lines screened. The results from selected lines are presented in Fig. III.4.

In general, the expected RNA and protein products were detected in the various transgenic plant lines examined. Transcript expression levels generally correlated with protein expression levels as determined by Western blot analysis. Different expression levels between different transgenic plant lines were also noted.

Inoculation of transgenic plants with virus. To estimate virus resistance in transgenic plants expressing different forms of the TEV CP, I challenged plants with TEV. Typically, inoculation of Burley 49 plants with TEV (either purified virus or plant sap) resulted in severe chlorosis and mosaic and mottle on systemically infected leaves approximately 6-7 days after inoculation. Severe etching of the leaf followed within a few days. I observed that transgenic plants containing only the CaMV promoter and untranslated sequences (i.e. the 35S plant line) responded to challenge inoculation in a manner similar to wild type Burley 49, developing extensive chlorosis and etching at the same rate (Fig. III.5 and Fig. III.6, B and C). Plant lines that expressed either FL or ΔN29 forms of TEV CP showed little, or no delay in the appearance of symptoms when inoculated with infected plant sap (Fig. III.5, A and C). However, FL and ΔN29 transgenic plants did show a slight attenuation of symptoms and eventually (2-4 weeks after initial appearance of symptoms) younger leaf tissue emerged devoid of symptoms and virus as demonstrated by back inoculation experiments (data not shown). Typically chlorosis and etching on older systemic leaves was limited (Fig. III.6 E and F). In contrast transgenic plant lines which expressed ΔC18 or ΔN/C forms of TEV CP showed significant delays in the appearance

Fig. III.4. Western blot analysis of transgenic plants. Protein samples (50 μg per lane) from leaf tissue of transgenic plants were separated on a 12.5% polyacrylamide gel containing SDS, and transferred to nitrocellulose. After blocking with 3% gelatin, bound antigen was detected with rabbit anti-TEV polyclonal antisera, goat anti-rabbit (alkaline phosphatase conjugate) antibody and the chromogenic substrates NBT and BCIP. Ten nanograms (ng) of purified TEV was used as a size marker (lanes 2 and 15, labeled on left and right sides of the Figure). Specific degradation of TEV CP occurs during virus purification and repeated freezing/thawing resulting in a doublet of 30 and 27 kDa and a smaller, fainter band of about 24 kDa. Lanes 1-15 contain protein samples from the following transgenic plant lines: 35S #4 (lane 1), 35S #4 plus 10 ng TEV (lane 2), FL #3 (lane 3), FL #24 (lane 4), ΔC18 #7 (lane 5), ΔC18 # 15 (lane 6), ΔN29 #1 (lane 7), ΔN29 #2 (lane 8), ΔN29 #8 (lane 9), ΔN/C #69 (lane 10), ΔN/C #61 (lane 11), AS #3 (lane 12), RC #5 (lane 13), 35S #4 (lane 14), 35S #4 plus 10 ng TEV (lane 15).

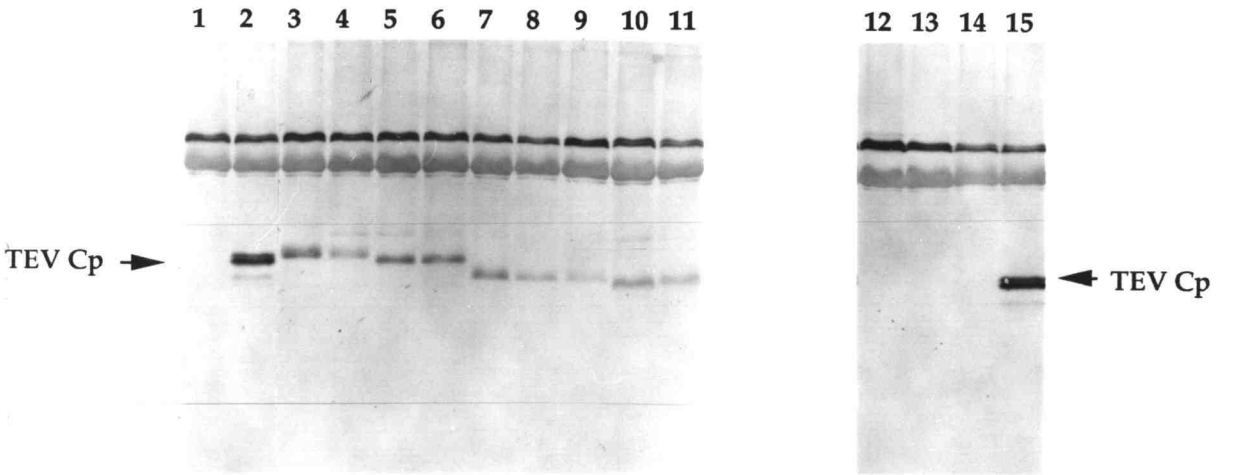


Figure III.4

Fig. III.5. Time of appearance of systemic symptoms in transgenic plants. Ten B49 (wild-type) plants and ten R2 plants of transgenic plant lines 35S #4, FL #3, FL #24 (**A**), ΔC18 #7, ΔC18 #15 (**B**), ΔN29 #1, ΔN29 #2, ΔN29 #8 (**C**), ΔN/C #69, and ΔN/C #61 (**D**), homozygous for the inserted TEV gene, were mechanically inoculated with 50 μl of 1:10 dilution of infected plant sap. Twenty B49 plants and 20 R1 plants of lines AS #3 and RC #5 were mechanically inoculated with 50 μl of 5 μg/ml TEV (**E**). Plants were evaluated daily and any plants displaying systemic symptoms (attenuated or wild-type) were recorded as symptomatic.

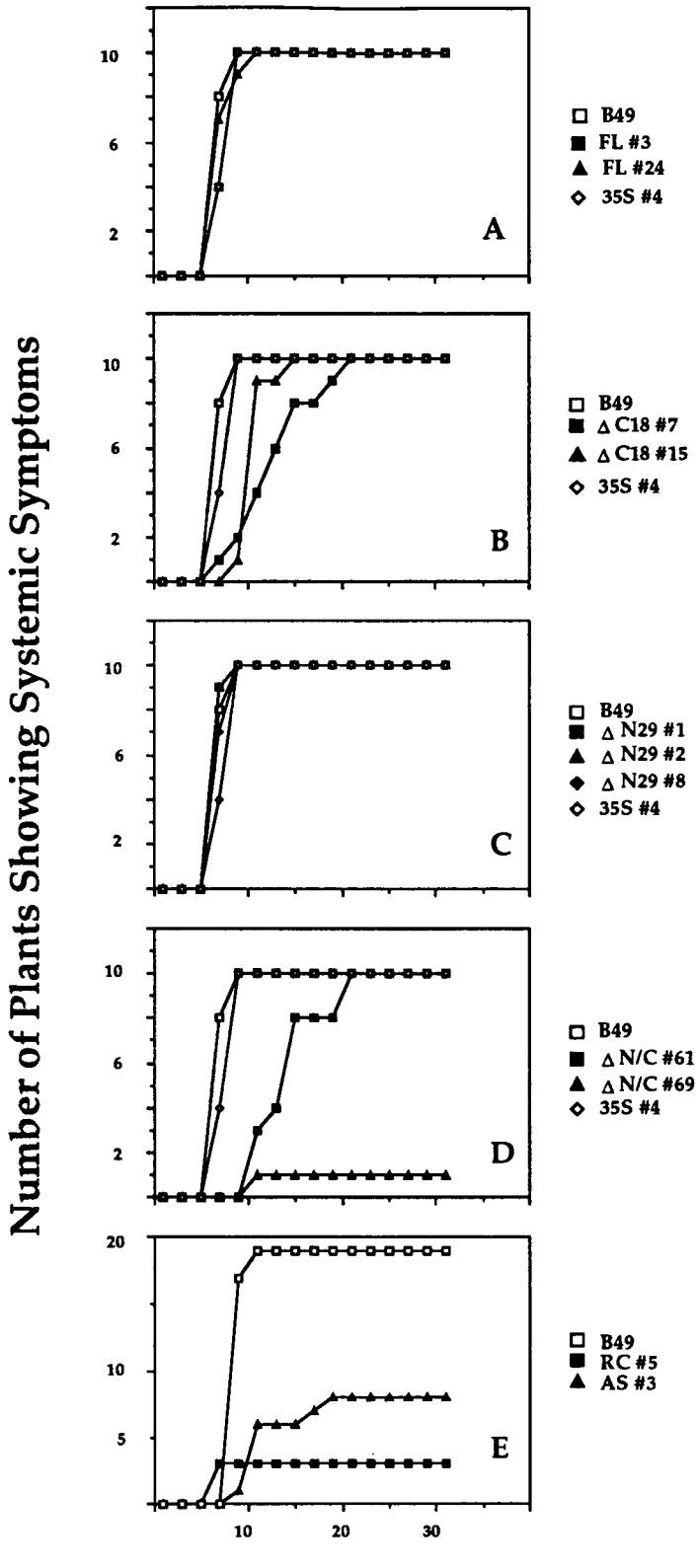

Number of Plants Showing Systemic Symptoms
10
6
2
A
B49
FL #3
FL #24
35S #4
10
6
2
B
B49
Δ C18 #7
Δ C18 #15
35S #4
10
6
2
C
B49
Δ N29 #1
Δ N29 #2
Δ N29 #8
35S #4
10
6
2
D
B49
Δ N/C #61
Δ N/C #69
35S #4
20
10
5
E
B49
RC #5
AS #3
10
20
30
Days Post Inoculation

Figure III.5.

Fig. III.6. Photographs of individual leaves of transgenic and wild type *Nicotiana tabacum* cv. Burley 49 uninoculated or inoculated with TEV. Individual leaves were removed from plants which had been inoculated with TEV 14 days earlier (or uninoculated Burley 49 plant) and photographed on a light box with transmitted light to illustrate symptom attenuation in transgenic plants. Symptoms in (**D**), (**G**), and (**H**) were not readily apparent without the use of transmitted light. Leaf samples were from (**A**) uninoculated Burley 49; (**B**) TEV-infected Burley 49; **C** through **H** show a TEV infected leaf from transgenic plant lines: (**C**) 35S #4; (**D**) AS #3; (**E**) FL #3; (**F**) ΔN29 #2;(**G**) ΔC18 #7; and (**H**) ΔN/C #69.

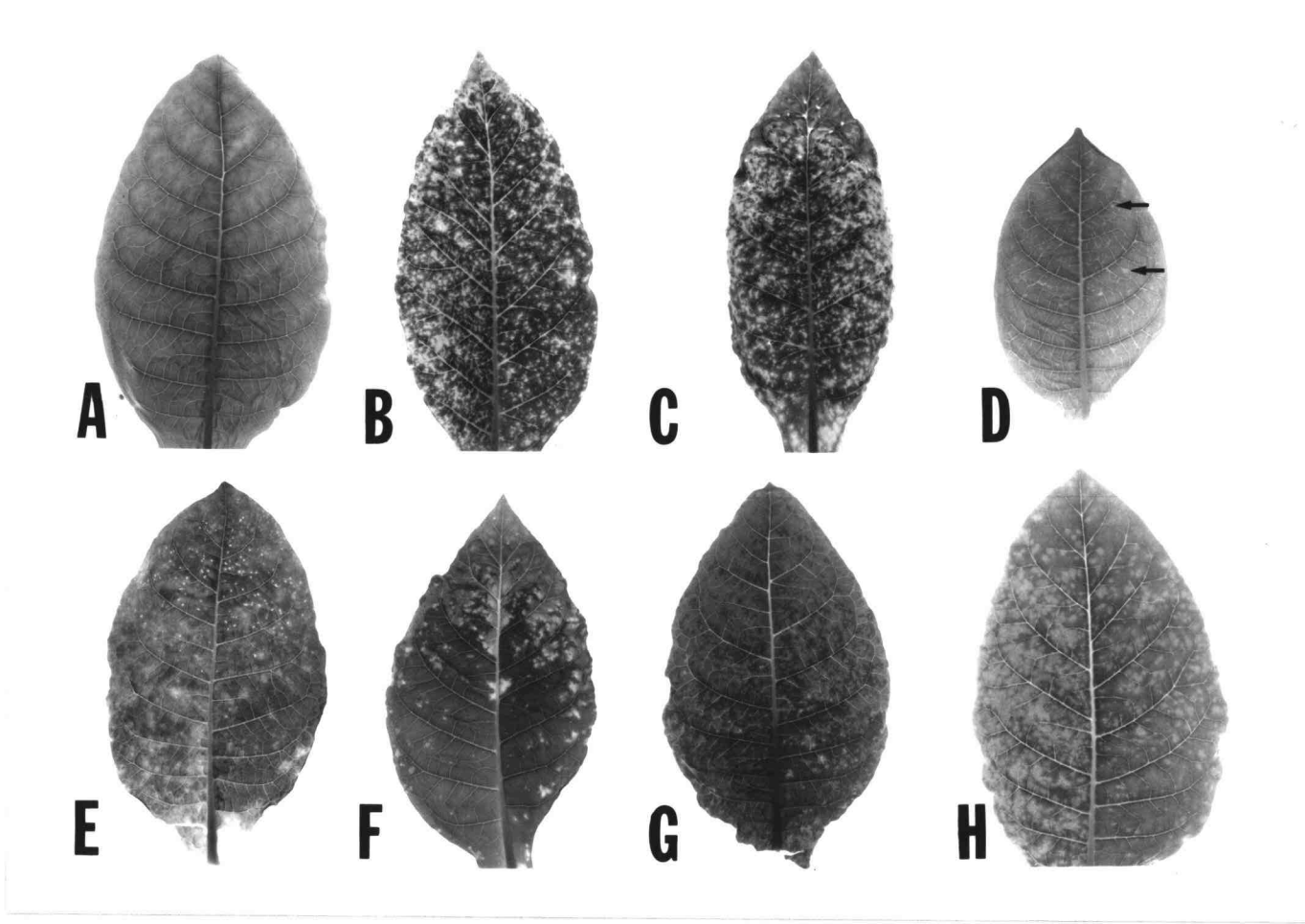


Figure III.6

of systemic symptoms (Fig. III.5, B and D). ΔC18 and ΔN/C plant lines also showed a pronounced modification of symptoms in systemically infected leaves as only a mild mosaic was observed (note lack of etching in Fig. III.6 G and H). However, ΔC18 and ΔN/C plants did not completely outgrow symptoms and a mild mosaic was detected on most leaf tissue.

Transgenic Burley 49 plant lines AS#3 and RC#5, expressing only TEV CP related RNA sequences, showed a delay in the appearance of symptoms, (Fig. III.5, E) and a modification of symptoms when inoculated with TEV. Since the 20 R1 plants were not screened for expression of CP RNA prior to inoculation, some of the symptomatic plants represented non-expressing plants in which the gene of interest had been lost during Mendelian segregation. Modified symptoms on AS#3 plants appeared as small chlorotic lesions often associated with a vein (see arrows in Fig. III.6, D). Most of the leaves were devoid of symptoms and virus (determined by back inoculation experiments). Approximately 15% of RC#5 plants showed symptoms which were identical to those of infected Burley 49. However, the remaining RC #5 plants were entirely asymptomatic and virus was not detected in back inoculation studies (data not shown).

Analysis of TEV replication in protoplasts derived from transgenic plant lines

I was surprised by the results obtained when AS and RC transgenic plants were challenged with TEV and by the alteration of symptoms observed with the different CP expressing plants. In my initial attempt to explain these results, I sought to determine if all of the transgenic plant lines I investigated would support virus replication at a level comparable to Burley 49. Accumulation of viral encoded proteins was used as an indirect

indicator of viral replication. Protoplasts were derived from leaf tissue of homozygous CP expressing plants and electroporated with TEV RNA. Four days after electroporation, protoplast proteins were extracted and assayed for the presence of TEV-encoded protein, indicative of TEV gene expression and replication, by Western blot analysis. These results, presented in Fig. III.7, indicated that protoplasts from all CP-expressing plant lines (full length and truncated versions) supported virus replication at levels comparable to wild type Burley 49 protoplasts. R1 transgenic plants from lines AS#3 and RC#5 were initially screened by Northern analysis and leaves from positive expressors were used in the production of protoplasts. Transfected protoplasts derived from AS#3 plants supported TEV replication, albeit at a reduced level. Protoplasts derived from RC #5 transgenic plant leaf tissue did not support TEV replication at a detectable level. These results (Fig. III.7), and those presented in the whole plant inoculation series (Fig. III.5, E), suggested AS and RC plants interfere with TEV replication.

Fig. III.7. Western blot analysis of tobacco etch virus (TEV) RNA transfected protoplasts. Protoplasts (1×10^{6}) were electroporated in the presence (lanes marked +) or absence (lanes unmarked) of 6 μg of purified TEV RNA. After 94 hours, protoplasts were collected and extracted by grinding in 2X Laemmli (1970) Tris-glycine running buffer. The equivalent of 5×10^{4} viable protoplasts were loaded per lane. Proteins were separated by electrophoresis and TEV proteins detected by Western blotting conditions identical to those described in Fig. III.2. Migration of TEV CP in this analysis, indicated on the left and right, was determined by adding 10 ng of purified TEV to a sample of 5×10^{4} B49 protoplasts (lanes 1 and 30). Electroporated protoplasts were derived from the following tobacco lines: B49 (lanes 2, 3, 24 and 25), FL #3 (lanes 4 and 5), FL #24 (lanes 6 and 7), ΔC18 #7 (lanes 8 and 9), ΔC18 #15 (lanes 10 and 11), ΔN29 #1 (lanes 12 and 13), ΔN29 #2 (lanes 14 and 15), ΔN29 #8 (lanes 16 and 17), ΔN/C #69 (lanes 18 and 19), ΔN/C #61 (lanes 20 and 21), 35S #4 (lanes 22 and 23), AS #3 (lanes 26 and 27), and RC #5 (lanes 28 and 29).

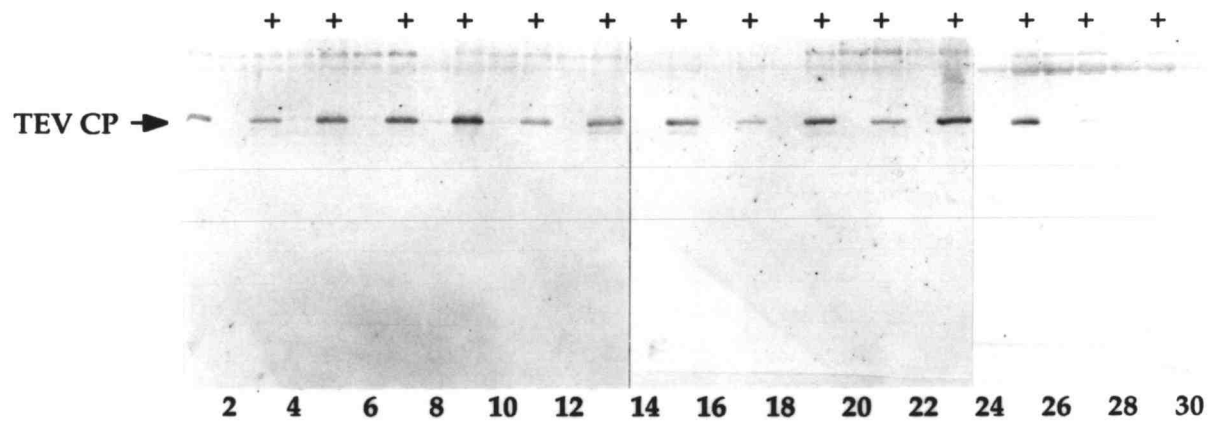


Figure III.7

Discussion

I have generated transgenic plants expressing either full-length CP, amino-terminal truncated CP, carboxy-terminal truncated CP, or amino- and carboxy-terminal truncated CP of TEV. The amino- and carboxy-termini of potyvirus CPs reside on the exterior of intact virions. Comparisons between different potyvirus CP nucleotide sequences has revealed that the amino- and carboxy- termini are highly variable in size and sequence while the internal "core" CP amino acids tend to be very conserved. Our ΔN29, ΔC18, and ΔN/C truncations maintain the highly conserved TEV CP core (Allison *et al.*, 1985a; Dougherty *et al.*, 1985; Shukla *et al.*, 1988). I sought to determine if these truncated versions of the CP gene would be functional in CP-mediated resistance.

My studies suggested that transgenic plants expressing mutated TEV CPs (either ΔC18 or ΔN/C forms, in particular) were more effective in CP-mediated resistance to TEV than transgenic plants which expressed FL TEV CP. I speculate that the truncated CPs are in some way dysfunctional and are more effective at disrupting the normal virus-host relationship than FL CP. This disruption may be at the level of virus movement throughout the plant. There have been reports of CP deletions in tobamovirus systems which affect virus movement (Dawson *et al.*, 1988). In these studies, viral genomes encoding truncated CPs are dysfunctional in the process of systemic (long distance) movement. Perhaps in the TEV system, truncated CP molecules are dysfunctional in the process of long distance movement and, as a consequence of being incorporated into virions, generated defective virus or ribonucleoprotein complexes inhibited in systemic movement. I have not yet investigated the affect of truncated CPs on short distance

movement. The reported "broad spectrum resistance" (Stark and Beachy, 1989; Ling *et al.*, 1991) for potyviruses may also involve this phenomenon. CPs from heterologous potyviruses may protect because they are dysfunctional in the heterologous system, resulting in limited systemic movement of the virus.

This study represented my initial analysis of transgenic plant lines. My goal is to select 12-15 independent lines of each construct to complete our germplasm bank. Although a limited number of transgenic plants have been tested to date, in general it appears as though ΔC18 coat proteins (either ΔC18 or ΔN/C forms) confer more effective resistance to virus than transgenic plants which express either full-length or ΔN29 truncated coat proteins. To my knowledge this was the first report of CP-mediated resistance in which truncated CP forms demonstrate significant virus resistance. Since protoplasts from all CP expressing transgenic plants examined support virus replication, subsequent efforts to unravel the mechanism involved in the observed resistance will focus on how CP expressing transgenic lines might primarily interfere with systemic movement of the replicating virus.

Transgenic plants expressing AS RNAs showed a delay in symptoms and a reduction in symptom severity (AS #3 plants). Transgenic plant line RC #5 was apparently immune to TEV. Protoplasts derived from AS#3 transgenic leaf tissue did not support TEV replication at wild-type levels, while protoplasts from RC #5 did not support detectable TEV replication (Fig. III.7). I suggest that TEV resistance in these plants was due primarily to interference with a step in viral replication. My results with these two transgenic plant lines appear to be similar to those reported by Golemboski *et al.* (1990). They have reported that plants which express an RNA sequence

from the 3' end of the 180 kDa gene of tobacco mosaic virus (TMV), but no detectable protein, were highly resistant to TMV infection. There are other examples reported in which transgenic plant lines are highly resistant to challenge virus inoculation, yet the expected protein product is not observed (Kawchuk *et al.*, 1990, 1991). It may be that in these instances, as with the AS #3 and RC#5 plant line, the resistant phenotype is mediated through a defective RNA species and not the expected translation product, as demonstrated in the extensively studied TMV-transgenic tobacco system (Powell *et al.*, 1990). In the AS and RC plant lines, AS and RC RNA molecules may be interfering with viral replication by (1) hybridizing to + or - sense genome length TEV RNA (respectively), thereby interfering with transcription and/or translation; (2) binding to an essential host or viral factor (e.g. replicase); or (3) interfering with virion assembly. Further studies will focus on these questions. From these preliminary studies it appears that transgenic plants expressing defective RNAs or proteins will be among the most effective potyvirus control strategies.

At present, literature regarding CP-mediated resistance to potyviruses is limited. Two previous studies have investigated the ability of a potyvirus CP to confer resistance to a heterologous potyvirus (referred to as "broad spectrum resistance") (Stark and Beachy, 1989; Ling *et al.*, 1991). The only other report of potyvirus CP-mediated resistance involved transgenic potato plants expressing PVY CP (Lawson *et al.*, 1990). In this study a transgenic line (303) which expressed little, if any, PVY CP was highly resistant to PVY infection, while other transgenic lines expressing higher levels of PVY CP were more sensitive, showing only mild protection. In the experiments I have observed that transgenic lines expressing FL TEV CP show little or no protection to TEV challenge while plant lines which accumulate RNA, but

no CP, are highly resistant (AS#3 and RC#5 lines). It may have been that in the study of Lawson *et al.* (1990) a spontaneous mutant which resulted in an untranslatable PVY CP RNA was responsible for the high degree of PVY resistance observed in the absence of PVY CP.

In summary, I have developed a series of transgenic plant lines which express various forms of the TEV CP gene sequence. Delay in symptom formation and altered symptom phenotype correlated with the TEV sequence being expressed. AS and RC transgenic plant lines displayed a high level of resistance and in one case, immunity. The level of protection achieved in these plants may represent a new and effective way of generating potyvirus resistant germplasm. Collectively, this work would appear to validate the concept of pathogen-derived resistance (Sanford and Johnston, 1985) as a viable approach to the development of potyvirus resistant plants.

CHAPTER IV

UNTRANSLATABLE TRANSCRIPTS OF THE TOBACCO ETCH VIRUS COAT PROTEIN GENE SEQUENCE CAN INTERFERE WITH TOBACCO ETCH VIRUS REPLICATION IN TRANSGENIC PLANTS AND PROTOPLASTS

Abstract

Transgenic tobacco plants which express untranslatable sense or antisense forms of the tobacco etch virus potyvirus (TEV) coat protein (CP) gene sequence have been generated. One of seven transgenic plant lines expressing a CP gene antisense transcript showed an attenuation of symptoms when inoculated with TEV. Three of 10 transgenic plant lines expressing untranslatable sense stranded transcripts did not develop symptoms when inoculated with TEV. These lines were resistant to either aphid- or mechanically-transmitted TEV. In contrast to CP-mediated resistance reported for other viruses, resistance was (1) mediated by an RNA molecule; (2) TEV specific (i.e. "broad-spectrum resistance" was not observed); (3) independent of inoculum levels; (4) not dependent on plant size and; (5) due to decreased levels of virus replication. Protoplast experiments were used to demonstrate that resistant plant lines did not support the production of virus protein and progeny virus at wild-type levels.

Introduction

Potyviruses are members of the picornavirus-like supergroup of viruses (Strauss *et al.*, 1990). Members of this supergroup contain similarly organized RNA genomes and employ a gene expression strategy involving proteolytic processing of viral polyproteins. Potyviruses are flexuous rod-shaped viruses with an RNA genome of ca. 10,000 nt with a 5' covalently linked viral encoded protein (VPg) and a 3' polyadenylate sequence (reviews, see Dougherty and Carrington, 1988; Riechmann *et al.*, 1992). The RNA contains a single, large open reading frame (ORF) coding for a polyprotein of ca. 350,000 daltons. No subgenomic mRNAs are produced during the virus replication cycle (Dougherty, 1983).

It has been proposed that a normal host-pathogen relationship can be disrupted, leading to resistance, if the host organism expresses a pathogen-derived gene product which interferes with the pathogen's normal replicative cycle (Sanford and Johnston, 1985). This phenomenon, termed pathogen derived resistance (PDR), has been demonstrated for numerous plant virus groups. Transgenic plants accumulating a plant virus CP frequently are resistant to infection by the homologous virus. This form of PDR is termed CP-mediated resistance and has been demonstrated for a large number of plant viruses (reviews, see Beachy *et al.*,1990; Gadani *et al.*, 1990). Most examples of CP-mediated resistance constitute a delay in the appearance of symptoms (Beachy *et al.*, 1990). Interference with virus uncoating or cell-to-cell movement may be at least partly responsible for this phenomenon (Register and Beachy, 1988; Osbourn *et al.*, 1989; Wu *et al.*, 1990). Other examples of PDR directed at plant viruses involve the expression of nonstructural protein gene sequences (Golemboski *et al.*, 1990;

Carr and Zaitlin, 1991), sequences present in untranslated regions (Morch *et al.*, 1987) or various antisense RNAs (Hemenway *et al.*, 1988; Rezaian *et al.*, 1988; Cuozzo *et al.*, 1988; Powell *et al.*, 1989; Kawchuck *et al.*, 1991).

I have recently generated and described a series of transgenic plants which express either full-length or truncated forms of the TEV CP (Lindbo and Dougherty, 1992a). Attenuation and alteration of symptoms, following infection with TEV, was dependent on the particular form of CP-derived transgene expressed *in planta*. I have also noted, in preliminary studies, that certain transgenic plant lines expressing untranslated versions of the TEV CP gene sequence were resistant.

In this chapter I report the results of my analysis of a group of transgenic plants which express untranslatable sense (RNA Control, RC plant lines) or antisense (AS plant lines) CP transcripts. Only one of seven AS transgenic plant lines studied showed an attenuation of TEV replication in protoplasts and virus resistance in whole plants. Three of ten RC transgenic plant lines examined did not display symptoms when inoculated with TEV. Protected plant lines were resistant to both aphid- and mechanically-transmitted TEV, but were sensitive to the closely related potyviruses potato virus Y (PVY) and tobacco vein mottling virus (TVMV). Protoplasts from resistant RC lines did not support the production of TEV antigen or infectious particles. A model is proposed to account for these observations.

Materials and Methods

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and used according to manufacturer's instructions. Plasmids were maintained in *Escherichia coli* strain HB101. Radiolabelled CTP [α-^{32}P] was purchased from New England Nuclear, Westwood, MA. T7 and SP6 RNA polymerases were purchased from Promega, Madison, WI.

Virus isolates used were obtained from the following sources: tobacco vein mottling virus (TVMV) and the TEV-H (highly aphid-transmissible) isolate were obtained from Dr. Tom Pirone, University of Kentucky; TEV-N (non aphid-transmissible) isolate was obtained from Dr. E. Heibert, University of Florida; TEV-S (severe) isolate and potato virus Y (PVY-N) were obtained from Dr. Guy Gooding, North Carolina State University. The TEV-OX isolate, originally isolated from infected pepper in Oxnard, CA, was obtained from Dr. Dan Purcifull, University of Florida. All viruses were maintained in *Nicotiana tabacum* cvs. Burley 49 (B49) or Burley 21.

Generation of AS and RC gene constructs

The construction of transgenic plant lines expressing AS, RC, or full length (FL) TEV CP gene constructs has been described in detail (Lindbo and Dougherty, 1992). Pertinent features of the transgene constructs in 35S, FL, AS, RC and 2RC plant lines and the nomenclature of the generated transgenic plants are presented in Fig. IV.1.

Fig. IV.1. TEV coat protein (CP) coding sequences used in generating transgenic plants. A cDNA sequence of the TEV CP gene was cloned into wide host range (WHR) vectors in either of two orientations. The resulting WHR plasmids were used in generating transgenic plants which would express either an antisense form of the TEV CP RNA (AS plant lines) or a translatable sense form of the CP RNA (FL plant lines). A mutated form of the TEV CP sequence, which would express an untranslatable sense form of the TEV CP RNA (as in RC and 2RC plant lines), was also cloned into the WHR vector. Transgenic plants transformed with WHR vector sequences only were generated as controls (35S plant lines). The nucleotide sequence in the region of the mutations is shown. Mutated nucleotides are in bold, enlarged type and the frameshift mutation is shown in superscript form. Four of the mutations generate stop codons, and one mutation changes the second codon from GGC to GCC. The arrow heads represent the location of the stop codons (underlined) generated in the CP open reading frame (ORF) in RC constructs. Abbreviations used in this diagram are as follows: CaMV, cauliflower mosaic virus; Enh 35S, "enhanced" CaMV 35S promoter; UTS, untranslated sequence; TEV, tobacco etch virus; TML, tumor morphology large.

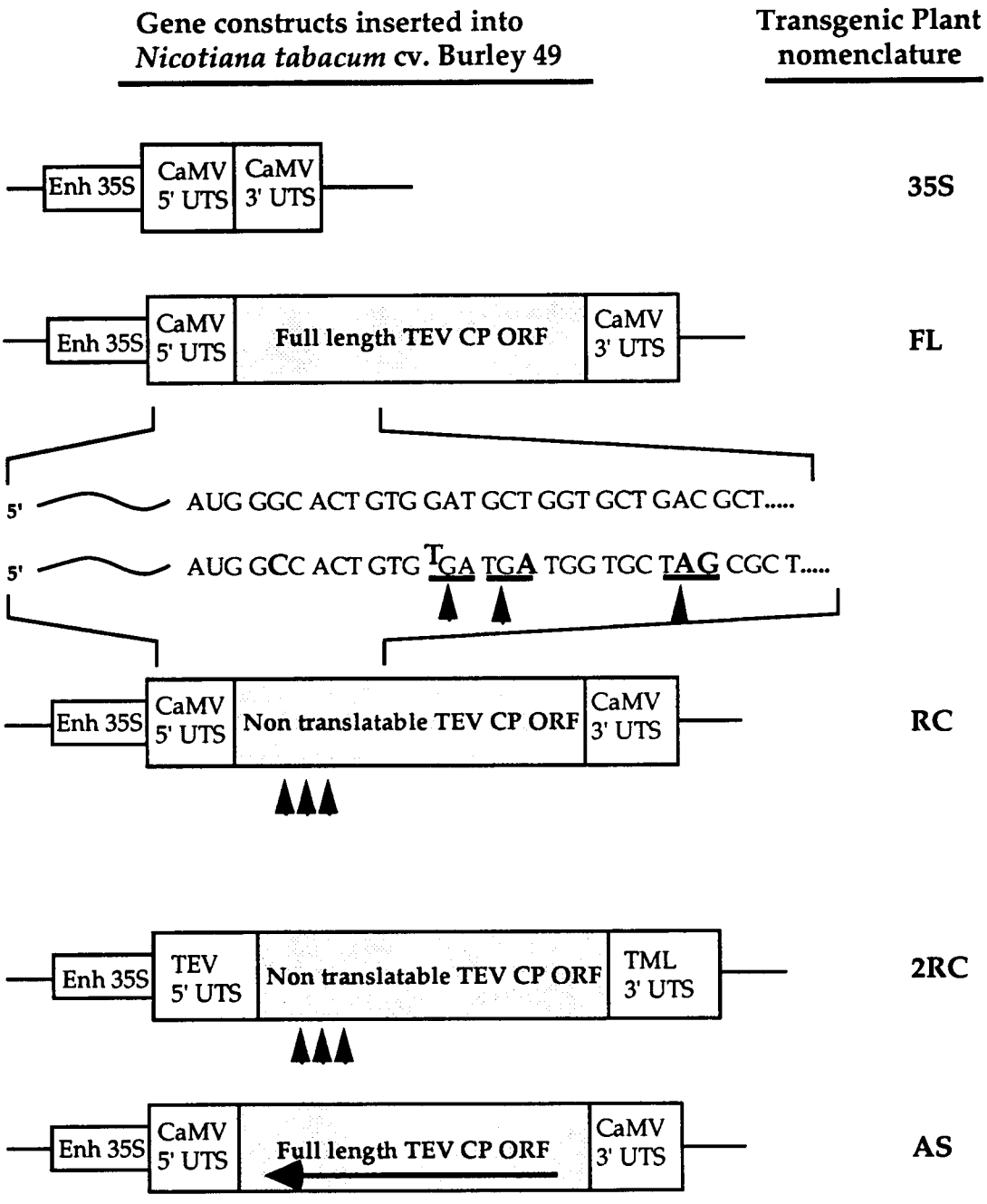

Gene constructs inserted into
Nicotiana tabacum cv. Burley 49
Transgenic Plant
nomenclature
Enh 35S
CaMV
5' UTS
CaMV
3' UTS
35S
Full length TEV CP ORF
FL
5'
AUG GGC ACT GTG GAT GCT GGT GCT GAC GCT.....
AUG GCC ACT GTG TGA TGA TGG TGC TAG CGC T.....
Non translatable TEV CP ORF
RC
TEV
5' UTS
TML
3' UTS
2RC
AS

Figure IV.1.

Wide host range plasmids containing T-DNA borders, TEV CP sequences (Fig. IV.1.) and a neomycin phosphotransferase II (kanamycin resistance) selectable marker were mobilized from *E. coli* into *Agrobacterium tumefaciens* A136 as described (Lindbo and Dougherty, 1992a).

Plant transformation

Leaf discs of *Nicotiana tabacum* cv. B49 were transformed and whole plants regenerated according to the procedures of Horsch and co-workers (1985). Transformed tissue was selected in the presence of 100 μg/ml kanamycin sulfate. Callus, shooting, and rooting media were as described (Lindbo and Dougherty, 1992a).

Analysis of transgene expression

Selected R1 and R2 generation transgenic plants were analyzed for expression of the transgene. Total nucleic acids were isolated from leaf tissue and RNA precipitated with LiCl as described in Verwoerd *et al.* (1989). Northern blotting conditions were as described in Lindbo and Dougherty (1992a). Strand specific ^{32}P-labeled RNA probes generated from a cDNA clone of the TEV CP region were used to detect plus or minus sense transcripts.

RNA slot blot analysis (Sambrook *et al.*, 1989) was used to estimate levels of transgene expression in R2 generation transgenic plants. Plus or minus sense TEV CP transcripts were used in generating a standard curve. Duplicate slot blots were probed with a strand specific TEV CP RNA probe or an actin specific RNA probe as an internal standard. Nitrocellulose filters

were scanned using an Ambis Radioanalytic Imaging System (AMBIS Systems Inc., San Diego, CA) to quantitate the amount of bound label.

In vitro transcription reactions

Both plus and minus sense TEV CP transcripts were generated from plasmid pTL 37/8595 (Carrington and Dougherty, 1987). Plasmid pTL 37/8595 contains a cDNA copy of the TEV CP gene flanked by T7 and SP6 RNA polymerase promoters. T7 or SP6 *in vitro* transcription reactions (Melton *et al.*, 1984) of linearized pTL 37/8595 as templates were used to synthesize unlabeled marker transcripts or ^{32}P-labeled RNA probes as described in Lindbo and Dougherty (1992a). The actin specific RNA probe was generated from a partial actin cDNA sequence (obtained from Dr. Russ Meints, Oregon State University) cloned into the vector pGEM-3 (Promega Corp., Madison, WI). RNA probes were synthesized using suggested procedures (Promega Corp., Madison, WI).

DNA Isolation and Southern Blot Analysis

Genomic DNA was isolated from leaf tissue using the procedure of Rogers and Bendich (1988). Purified DNA was digested with selected restriction enzymes and 10 μg of digested DNA analyzed by Southern blotting procedures (Southern, 1975), using TEV CP specific RNA probes.

Inoculation of Transgenic Plants

Generally, five to ten R2 generation plants per line per test were inoculated mechanically with infected plant sap. Plants were inoculated as seedlings (two true leaves ca. 2 cm in diameter) or when 5, 10 or 15 cm in height with various TEV isolates or with TVMV or PVY. Preparation of

inoculum and inoculation conditions were as described (Lindbo and Dougherty, 1992a). The TEV inoculum used could be diluted 1:5000 and still established a systemic infection on *N. tabacum* (B49 or B21). Inoculated plants were observed daily for symptoms.

Plants were also inoculated using viruliferous aphids (*Myzus persicae*). Aphids were fasted for 2 hr and acquired TEV by feeding for 3 min through stretched parafilm membranes on a mixture of purifed PVY helper component and purified TEV-H in a buffered sucrose solution (Govier *et al.*, 1977). PVY helper component was prepared as described by Govier and co-workers (1977) and TEV-H was purified using the method of Mohgal and Francki (1976). Ten feeding aphids were then transferred to each test plant and allowed to feed for ca. 12 hr. Alternatively, fasted aphids were allowed a 2-5 min access time on TEV-H infected B49 leaf tissue. Four feeding aphids were transferred to each test plant. Seedlings and plants 5 cm in height were used in these studies. Inoculated plants were observed daily for symptoms.

Preparation, inoculation and analysis of protoplasts

Protoplasts were prepared from transgenic tobacco plants and electroporated according to the procedure of Luciano *et al.* (1987) as modified in Lindbo and Dougherty (1992a). Protoplasts (1×10^6) were electroporated in the presence or absence of 6 μg of purified TEV RNA. Following electroporation, protoplasts were incubated for 96 hr in incubation media (Luciano *et al.*, 1987).

After a 96 hr incubation period, protoplasts were stained with fluoroscein diacetate to estimate viability (Widholm, 1972) and used in Western blot or back inoculation experiments. Protoplasts (2.5×10^4, viable) were extracted in 2X Laemmli (Tris-glycine, SDS) running buffer (Laemmli,

1970) and analyzed by Western blot analysis (Towbin *et al.*, 1979) as described in Lindbo and Dougherty (1992a). For back inoculation studies, 1×10^5 viable transfected protoplasts were ground in the presence of 100µl of 100 mM sodium phosphate buffer (pH 7.8) and the resulting extract used to inoculate two *N. tabacum* cv. B49 plants (50 µl per plant). Plants were observed daily for the appearance of symptoms.

Results

Production and analysis of transgenic plants

R1 generation AS and RC transgenic plants initially were screened by RNA dot blot analysis. Strand-specific RNA probes were used to identify transgenic plants expressing TEV specific RNA sequences (data not shown). Selected R1 generation transgenic plants were selfed and the resulting R2 progeny screened for expression of the kanamycin resistance ($Kan^{r)}$ marker and the TEV CP transgene sequence. R2 seedlings were screened for Kan^r (50 to 200 R2 seedlings per line) and for RNA expression (10 R2 plants per line) by dot blot analysis (data not shown). R2 generation plant lines which gave 100% positives in both of these tests were considered to be homozygous and used in subsequent experiments. This initial screening resulted in the identification of 7 AS and 10 RC lines (nine RC- and one 2RC-type transgenic plant lines) as homozygotes to be used in this study.

I asked if the transgenic plant lines generated were the result of single or multiple insertion events during *Agrobacterium*-mediated transformation. Transgene copy number was estimated by segregation of the Kan^r phenotype in R2 seed samples (see above), or by Southern blot analysis of transgenic plant genomic DNA. Genomic DNA was isolated from AS and RC transgenic plants, digested with various restriction enzymes, and analyzed by Southern blot procedures (Southern, 1975) to estimate copy number of the transgene in the genome. Most lines appeared to contain a single copy of the gene, although multiple copies were detected in six transgenic plant lines. The data are summarized in Table IV.1.

Table IV.1. Estimated Gene Copy Number and Expression Level in Transgenic Plants[a]

Sample	copy #	expression level (pg/μg)
AS #3	1	6.6
AS #4	1	1.8
AS #5	1	11.6
AS #6	1	4
AS #7	2	10.6
AS #9	2	20.6
AS #10	1	6.6
RC #1	1	1.4
RC #2	1	3
RC #3	ND	5.6
RC #4	1	3
RC #5	2	3
RC #7	3	3.8
RC #8	ND	9.4
RC #9	2	2.8
RC #10	ND	1.2
2RC #6	2	5.8

[a]Gene copy number was estimated by segregation of the kanamycin resistance phenotype and/or Southern blot analysis of genomic DNA isolated from transgenic plants. RNA expression levels were estimated by slot blot analysis of total RNA isolated from transgenic plants. Slot blots were probed with a strand specific TEV RNA probe. The expression levels presented are an average of two trials and are expressed as pg of transgene transcript/μg of total RNA. ND = not determined.

I also sought to characterize the RNA product of the introduced gene. Total RNA samples, isolated from transgenic tobacco plants, were analyzed in Northern blot hybridization experiments. Strand specific ^{32}P-labeled RNA probes were used to demonstrate that transgenic plants expressed transcripts of the expected size and "sense". A transcript of ca. 1,100 nucleotides (nt) was expected in RC and AS transgenic plants and a transcript of ca. 1,350 nt was expected in the 2RC line. The expected RNA transcripts were detected in the various transgenic plant lines examined. Fig. IV.2, A and B, show Northern blot analysis of RNA samples from various transgenic plants.

To quantify accumulated levels of the TEV-related transcript in the different transgenic plant lines, slot blot analysis of transgenic plant RNA was conducted. Various levels of transcript, ranging from ca. 1 to 20 pg CP transcript per microgram total RNA, were found in the different transgenic plant lines (Table IV.1.).

Inoculation of transgenic plants with virus

I sought to determine if these homozygous transgenic plant lines would display a resistant phenotype to potyvirus infection. To examine this, transgenic plants (ca. 15 cm in height) were challenged with sap extracts from plants infected with one of four TEV isolates (TEV-H, N, OX, or S) or with the potyviruses PVY-N or TVMV. Typically, *N. tabacum* cv. B49 plants inoculated with these viruses display symptoms approximately 7 days after inoculation. In our studies some plant lines were resistant to TEV. In replicated experiments, one AS line (AS #3) occasionally showed attenuated symptoms when inoculated with TEV isolates (data not shown). Plants of line RC #5 were generally resistant to TEV infection. However, a low

Fig. IV.2. Northern blot analysis of transgenic plant RNA. Total RNA was isolated from various transgenic plant lines and electrophoretically separated on 1.2% agarose gels containing 6% formaldehyde. Varying levels of RNA (between 1 and 5 μg) were loaded per lane so that bands could be clearly seen in all samples. Filters were hybridized with a ^{32}P-labelled RNA probe specific for minus sense (**A**), or plus sense (**B**) CP transcripts. After washing under stringent conditions (65 °C in 0.1X SSC, 2 hr), filters were exposed to Kodak X-Omat film. Autoradiograms of the blots are presented. Mobility and estimated length (in nt) of minus and plus sense marker transcripts containing the TEV CP sequence (A and B, lanes 1 and 21, respectively) are presented on the sides of the figure. Identity of transgenic plant lines from which RNA was extracted are labelled above autoradiograms. RNA from *N. tabacum* cv. Burley 49 (B49) is in lanes 2 and 20. Nomenclature for transgenic plant lines is as follows: (1) RC and AS describe if the plant line is expressing a untranslatable sense or antisense form of the TEV CP RNA, respectively, (2) the numbers following the AS and RC designation refer to separate transformation events which gave rise to transgenic plant lines.

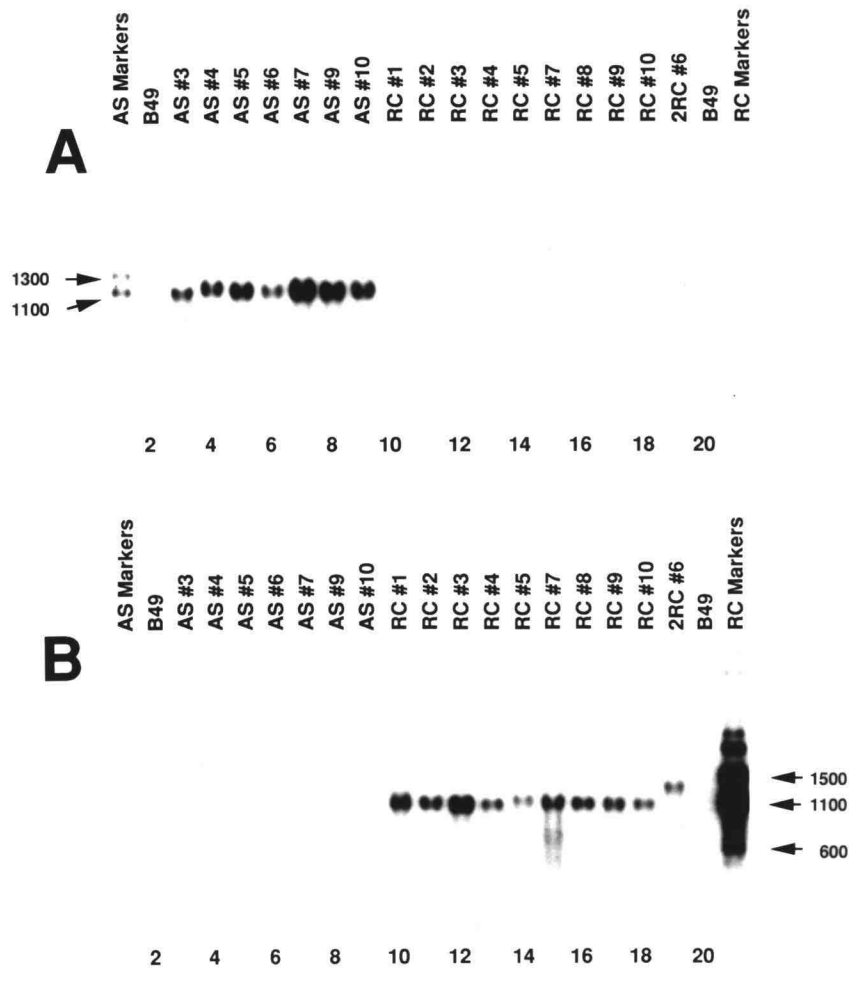

A
AS Markers
B49
AS #3
AS #4
AS #5
AS #6
AS #7
AS #9
AS #10
RC #1
RC #2
RC #3
RC #4
RC #5
RC #7
RC #8
RC #9
RC #10
2RC #6
B49
RC Markers
1300
1100
2
4
6
8
10
12
14
16
18
20
B
AS Markers
B49
AS #3
AS #4
AS #5
AS #6
AS #7
AS #9
AS #10
RC #1
RC #2
RC #3
RC #4
RC #5
RC #7
RC #8
RC #9
RC #10
2RC #6
B49
RC Markers
1500
1100
600
2
4
6
8
10
12
14
16
18
20

Figure IV.2.

percentage of such plants (less than 5%) did show symptoms when inoculated with various TEV isolates (Tables IV.2 and IV.3). In several experiments some plants of line RC #4 have not developed symptoms (Table IV.3). Transgenic plant lines RC #7 and 2RC #6 showed no symptoms when inoculated with any of the four TEV isolates. All other AS and RC transgenic plant lines exhibited wild type sensitivities to the TEV isolates used. No AS or RC plant lines displayed symptom attenuation when inoculated with PVY or TVMV (Table IV.2.). Experiments typically were terminated 3-4 weeks post inoculation.

Mechanical and aphid vectored inoculation of young plants

After determining that large (ca. 15 cm in height) plants of selected lines were resistant to mechanically applied inoculum, I sought to determine if younger transgenic plants were resistant to mechanically- or aphid-vectored TEV-H inoculum. To this end, I mechanically inoculated transgenic and control germplasm as seedlings (two true leaves ca. 2 cm in diameter) or as small plants (ca. 5 cm in height) with infected plant sap. I also attempted to transfer TEV to small plants and seedlings using viruliferous aphids. Results of these experiments were consistent with the results of the mechanical inoculation experiments involving plants ca. 15 cm in height. An occasional RC #5 plant would become infected and some RC #4 plants would not exhibit symptoms, while plants of lines RC #7 and 2RC #6 were completely refractory to TEV infection (Table IV.3). The other transgenic plant lines were susceptible to TEV infection.

TABLE IV.2.

Susceptibility of transgenic plant lines to potyviruses.[a]

Plant line	Virus Inoculum					
	TEV-H	TEV-N	TEV-S	TEV-OX	PVY	TVMV
B49	8/8	8/8	8/8	8/8	8/8	8/8
35S #4	8/8	8/8	8/8	8/8	8/8	8/8
FL #3	8/8	8/8	8/8	8/8	8/8	8/8
AS #3	8/8	8/8	8/8	8/8	8/8	8/8
AS #4	8/8	8/8	8/8	8/8	8/8	8/8
AS #5	8/8	8/8	8/8	8/8	8/8	8/8
AS #6	8/8	8/8	8/8	8/8	8/8	8/8
AS #7	8/8	8/8	8/8	8/8	8/8	8/8
AS #9	8/8	8/8	8/8	8/8	8/8	8/8
AS #10	8/8	8/8	8/8	8/8	8/8	8/8
RC #1	8/8	8/8	8/8	8/8	8/8	8/8
RC #2	8/8	8/8	8/8	8/8	8/8	8/8
RC #3	8/8	8/8	8/8	8/8	8/8	8/8
RC #4	8/8	8/8	8/8	8/8	8/8	8/8
RC #5	0/20*	0/20	0/20	2/20	8/8	8/8
RC #7	0/8	0/8	0/8	0/8	8/8	8/8
RC #8	8/8	8/8	8/8	8/8	8/8	8/8
RC #9	8/8	8/8	8/8	8/8	8/8	8/8
RC #10	8/8	8/8	8/8	8/8	8/8	8/8
2RC #6	0/8	0/8	0/8	0/8	8/8	8/8

[a] Transgenic and wild type plants ca. 15 cm. tall (ca. 8 wks old) were mechanically inoculated with 50 µl of a 1:10 dilution of infected plant sap extract. Data are presented as the number of symptomatic plants (at 14 days post inoculation) over total number inoculated.

Viruses used in this study are as follows; TEV-H, TEV-highly aphid-transmissible isolate; TEV-N, TEV non aphid-transmissible isolate; TEV-S, TEV severe isolate; TEV-OX, TEV oxnard isolate; PVY, potato virus Y; TVMV, tobacco vein mottling virus. Plant line nomenclature is as in Fig IV.1.

*A low percentage of the plants of line RC #5 show symptoms when inoculated with TEV isolates. A larger sample size was used to demonstrate this non-uniform response.

TABLE IV.3.

Susceptibility of transgenic plants to aphid (A) or mechanically (M) transmitted TEV-H. [a]

Plant Line	Seedlings		5 cm tall plants	
	M	A	M	A
B49	10/10	8/10	5/5	3/10
35S	10/10	4/10	5/5	5/10
FL #3	10/10	5/10	5/5	1/10
AS #6	10/10	3/10	5/5	1/10
AS #7	10/10	1/10	5/5	2/10
AS #9	10/10	6/10	5/5	5/10
RC #1	10/10	3/10	5/5	3/10
RC #4	6/10	3/10	3/5	2/10
RC #5	0/10	1/10	0/5	0/10
RC #7	0/10	0/10	0/5	0/10
RC #9	10/10	6/10	5/5	1/10
2RC #6	0/10	0/10	0/5	0/10

[a] Data is presented as the number of symptomatic plants (at 14 days post inoculation) over total number inoculated. Selected transgenic plant lines were challenged at the seedling stage (two true leaves, ca. 2 cm diameter) or as small plants (ca. 5 cm. tall) with either mechanically- (M) transmitted TEV (50 µl of a 1:10 dilution of infected plant sap), or aphid- (A) vectored virus. For seedlings, aphids were fasted and then fed on an in vitro transmission system composed of purified PVY helper component and purified TEV-H (highly aphid-transmissible isolate of TEV). Ten aphids were transferred to each test plant. Alternatively, for 5 cm. tall plants, aphids were fasted and then allowed a 2-5 min access time on a TEV-H infected N. tabacum cv. Burley 49 leaf (3 weeks post inoculation). Four feeding aphids were transferred to each test plant. Plant line nomenclature is as in Fig. IV.1.

Analysis of TEV replication in protoplasts derived from transgenic plants.

The resistance phenotype manifested by the RC and 2RC lines could be a function of inhibition of virus replication or cell-to-cell movement. An indirect test of this mode of action would be analysis of TEV replication in protoplasts derived from the various transgenic backgrounds. Protoplasts derived from wild-type and transgenic plants were electroporated in the presence or absence of purified TEV RNA and later assayed for the production of viral antigen (by Western blot analysis) and infectious particles (by back inoculation to B49 seedlings). Protoplasts from *N. tabacum* cv. B49 and transgenic plant line FL #3 support the production of viral antigen, and infectious particles (Table IV.4. and Lindbo and Dougherty, 1992a). The Western blot analysis of protoplast extracts suggested virus antigen (CP) accumulated to wild-type levels in protoplasts derived from the various transgenic plants with four notable exceptions. AS #3 derived protoplasts supported the production of viral antigen at a reduced level (Fig. IV.3 lane 3), but still produced infectious particles (Table IV.4). Protoplasts from resistant lines RC #5, RC #7 and 2RC #6 did not support production of detectable levels of CP (Fig. IV.3, lanes 19, 20, 24) or infectious particles (Table IV.4).

TABLE IV.4

Production of TEV antigen and infectious particles in transfected tobacco protoplasts [a]

Plant line	Viral antigen	Infectious particles
B49	+	+
35S	+	+
FL #3	+	+
AS #2	+	+
AS #3	+	+
AS #4	+	+
AS #5	+	+
AS #6	+	+
AS #7	+	+
AS #9	+	+
AS #10	+	+
RC #1	+	+
RC #2	+	+
RC #3	+	+
RC #4	+	+
RC #5	-	-
RC #7	-	-
RC #8	+	+
RC #9	+	+
RC #10	+	+
2RC #6	-	-

[a] Protoplasts derived from wild type (B49) and transgenic plant tissue were electroporated in the presence of 6 μg of purifed TEV RNA. Protoplasts were later assayed for production of viral coat protein by Western blot analysis, and for the presence of infectious particles through back inoculation experiments. In the above table, detectable levels of CP or infectious particles are represented by a "+". Protoplasts transfected in the absence of TEV RNA did not produce viral antigen or infectious particles (data not shown).

Fig. IV.3. Western blot analysis of TEV RNA transfected protoplasts. Protoplasts were obtained from both transgenic and *N tabacum* cv. Burley 49 plants. Protoplasts ($1x\ 10^6$) were electroporated in the presence of TEV RNA (6 μg) and placed in incubation media. After 96 hr 2.5×10^4 viable protoplasts were assayed for production of viral coat protein by Western blot analysis. Protoplast proteins were extracted in 2X Laemmli running buffer, separated on 12.5% polyacrylamide gels containing SDS and transferred to nitrocellulose. Rabbit anti-TEV primary antibodies and goat anti-rabbit alkaline phosphatase conjugated secondary antibody were used to detect bound antigen. The source of each protoplast sample is indicated above each lane. Transgenic plant nomenclature is as described for Fig IV.1. Lane 1 contains *N. tabacum* cv. Burley 49 (B49) plant extract with 10 ng of TEV added. Lanes 12 and 13 (B49 healthy) contained uninfected whole plant and protoplast samples, respectively. The migration of TEV CP is noted on the sides of the figure. The identity of the high molecular weight cross reacting bands is not known.

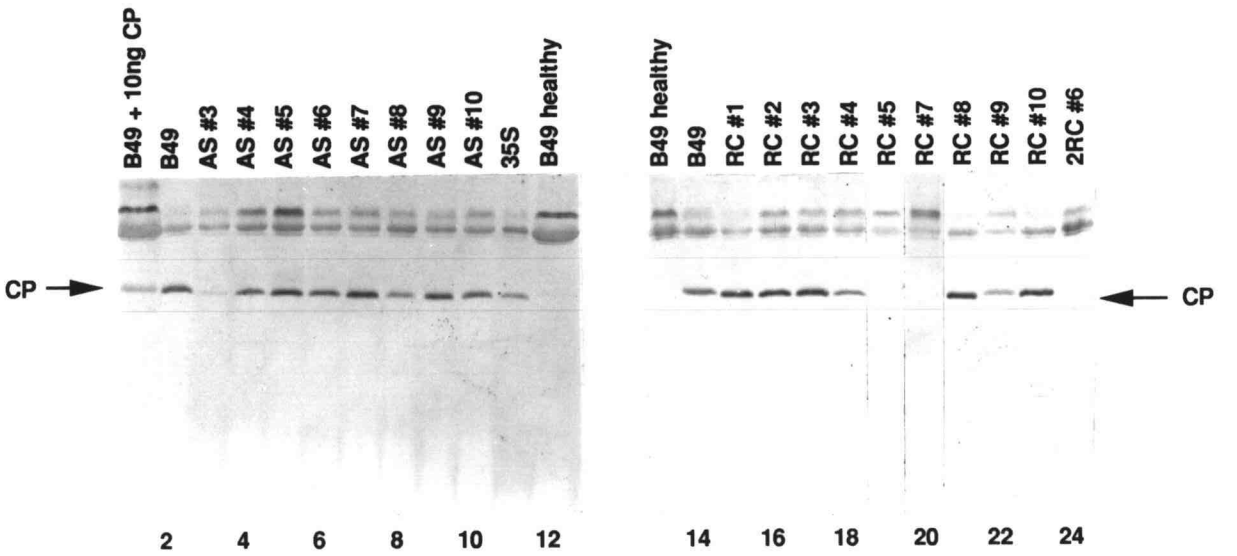

B49 + 10ng CP
B49
AS #3
AS #4
AS #5
AS #6
AS #7
AS #8
AS #9
AS #10
35S
B49 healthy
B49 healthy
B49
RC #1
RC #2
RC #3
RC #4
RC #5
RC #7
RC #8
RC #9
RC #10
2RC #6
CP
CP
2
4
6
8
10
12
14
16
18
20
22
24

Figure IV.3

Discussion

Transgenic plant lines expressing untranslatable TEV RNA can show high levels of resistance to TEV infection. I have developed a working model based on the transgene expressed by the various transgenic lines and my observations of the various germplasms in whole plant and protoplast studies. Central to my hypothesis is the RC transcript containing the stop codons immediately after the AUG initiator codon (Fig. IV.1) and the 3' untranslated region of the transcript. I have considered models in which the untranslatable RC transcript may participate in the resistance response by (1) forming an RNA/RNA hybrid with the minus strand TEV RNA species; (2) by binding factors (host or viral) essential for replication; or (3) by a combination of both interactions. Alternatively the transgene could have insertionally inactivated a host gene essential for TEV replication.

I do not favor the hypothesis involving the insertional inactivation of a host gene for two reasons. First, I would expect any inactivated host gene which affects TEV replication to also affect PVY and TVMV replication. This is not the case. Second, the resistance phenomenon has appeared in three independent transgenic plant lines with the transgene inserted into distinct genome locations. Therefore, I suggest insertional inactivation of an essential host gene would be unlikely.

I suggest the data are consistent with the RNA/RNA hybridization hypothesis similar to the RNA/RNA hybridization mechanism proposed to be involved in traditional cross protection (Palukaitis and Zaitlin, 1984). I propose that the annealing of RC-type transcripts to minus-sense genomes inhibits production of plus sense progeny RNA molecules by interfering

with the procession of replicase complexes on the minus strand template. During the course of a typical plus sense RNA virus replication cycle, sense strand genomes grossly outnumber minus sense molecules. For this reason, blocking the generation of plus sense genomes from minus sense templates may be a particularly vulnerable step in the viral replication cycle.

I propose that the RNA transcript from RC constructs maximizes the potential formation of an RNA/RNA hybrid. First, the three stop codons immediately after the initiator AUG should prevent ribosomes from translating the RNA. Second, there are no TEV 3' untranslated sequences and the TEV replicase complex would not be likely to bind or transcribe this RNA. Together, these features result in an RNA molecule which should have limited RNA/protein interactions that could interfere with RNA/RNA hybrid formation. In contrast, the FL gene construct produces an RNA transcript that only differs from the RC transcript by five nucleotides but is translated. I have never identified TEV coat protein producing lines (Lindbo and Dougherty, 1992a; unpublished results), including six different FL transgenic plant lines, which show the resistance phenotype associated with select RC lines. I propose that when ribosomes translate the RNA coding for TEV coat protein, they prevent RNA/RNA hybrid formation. Although I have not tested RC constructs with an authentic TEV 3' untranslated region, I would predict that TEV replication complexes could bind, potentially transcribe the RC RNA and, in the process, interfere with RNA/RNA hybrid formation.

The virus specific nature of the resistance is also consistent with the RNA/RNA hybridization model. Because of the high degree of nucleotide sequence homology between the four TEV isolates, the RC transcript would be expected to anneal extensively to genome length minus strand RNA of

these isolates. In the case of PVY and TVMV, however, the coat protein nucleotide sequences share 62% and 60% homology, respectively, with the TEV RC transcript. Therefore, the RC transcript would not be expected to form a stable RNA/RNA hybrid with the minus-strand RNA of these viral genomes and would not inhibit replication of these viruses.

Currently, I do not favor the model involving the transgene RNA of either viral or host replication factors, for the following reasons: (1) The RC construct lacks a TEV 3' untranslated region likely to contain some or all of the signals detected by viral factors essential for replication; (2) RC and FL lines only differ by five nucleotides yet the resistance phenotype has never been observed in FL plant lines; (3) the resistance is quite virus specific. However, the possibility of viral proteins binding to the CP ORF cannot totally be discounted, as the presence of regions other than 5' and 3' untranslated regions may also be involved in viral replication. In regards to the specificity of the resistance, since the potyviruses TEV, TVMV, and PVY are so closely related, we propose that these viruses would employ common host factors during replication. Therefore, if the RC transcript is binding such a host factor(s) and inhibiting replication of TEV, I would expect replication of PVY and TVMV to be attenuated as well.

Not all RC transgenic lines express the resistance phenotype, and resistance does not appear to correlate with RNA expression levels. Therefore, I must qualify my model with other observations made in this study. Additional undefined factor(s) must play a role in determining the resistance phenotype. For example, cell-type specific expression levels or subcellular localization of the untranslatable transcript may be important. It may be that in order for a plant to be protected, a basal level of expression is required in all cell types of that transgenic plant. All transgenic plants may

not express the transgene in all tissues. For example, Barnes (1990) has shown that different patterns of tissue specific expression can be obtained in transgenic plants transformed with the same gene construct. This may account for the difference in resistance observed with different RC lines. Identification of the factors or cellular conditions required for the RC transcript to function in resistance is the focus of our current studies.

My results differ significantly from the often-reported CP-mediated resistance. Much of the information regarding CP-mediated resistance involves the extensively studied TMV system (review, see Beachy *et al.*, 1990), though examples of CP-mediated resistance have been reported for potyviruses as well (Stark and Beachy, 1989; Lawson *et al.*, 1990; Ling *et al.*, 1991). The RC RNA-mediated protection results in plants which (1) do not display symptoms, even at high virus inoculum levels (infected plant sap) or when TEV RNA is used as inoculum; (2) show resistance in very young plants; (3) are not protected against challenge inoculation with heterologous viruses; and (4) mechanistically involves an attenuation of virus replication. The high degree of resistance obtained by this method makes it an attractive alternative to CP-mediated resistance.

Recently, other examples of transgenic plants expressing viral-derived genes, but no detectable levels of protein, have been published. These experiments have reported mixed results. Only one of these studies (Powell *et al.*, 1990) involved a gene construct designed to produce an untranslatable RNA. In this case, Powell and colleagues (1990) demonstrated that the TMV CP and not RNA was necessary for TMV resistance in transgenic plants. In contrast, in an attempt to demonstrate CP-mediated resistance to potato leaf roll virus (PLRV), Kawchuck and co-workers (1991) could not detect the production of PLRV CP in their

transgenic plants. However, these plants were resistant to PLRV vectored by viruliferous aphids. Golemboski and co-workers (1990) reported that transgenic plants expressing an RNA which would be translated into the TMV (putative) 54 kDa protein were resistant to one isolate of TMV. An RNA transcript from the transgene was present, but the 54kDa protein could not be detected in transgenic plants. TMV replication, however, was subsequently shown to be dramatically impaired in these plants (Carr and Zaitlin, 1991). These workers have suggested that the transgene RNA may be the basis of the resistance phenotype observed. However, in these studies, the constructs were designed to produce translatable RNAs and it is possible that undetectable levels of the expected protein may be responsible. In my TEV RC study, the gene construct was designed to produce an untranslatable RNA and, therefore, I suggest RNA is the effector molecule.

I suggest that this strategy will be successful, not only toward other potyviruses but with other viral systems as well. If the hypothesis is correct, multiple regions of viral genomes should be able to confer resistance, though some genomic regions may be more effective than others. For example, intramolecular base pairing of the untranslatable sense transcript and/or that of the minus sense genome-length RNAs could limit efficient hybridization between the two RNA species. Additionally, in selecting regions of the minus sense genome to target, features which maximize RNA/RNA hybrid formation should be emphasized. I suggest that 5' and 3' untranslated regions, and internal promoter binding sites, all conventional targets of antisense strategies, will not be effective. These regions of the genome likely participate in extensive protein/RNA binding interactions. Likewise, in virus systems which produce large quantities of subgenomic mRNAs, regions of the minus sense genome which are actively transcribed

during subgenomic mRNA production would not be favorable targets. Heavily transcribed regions of minus sense genomes are likely to be occupied by transcription complexes which would destabilize RNA/RNA hybrids.

In summary, I have generated transgenic plants which express untranslatable sense RNAs which interfere with viral replication. Although I have not yet determined all the experimental parameters or the exact mechanism of this inhibition I suggest this approach will complement CP mediated strategies and other traditional resistance genes.

CHAPTER V

INDUCTION OF A HIGHLY SPECIFIC ANTI-VIRAL STATE IN TRANSGENIC PLANTS: IMPLICATIONS FOR GENE REGULATION AND VIRUS RESISTANCE.

Abstract

Transgenic *Nicotiana tabacum* plants expressing either a full length or an amino-terminally truncated form of the tobacco etch virus (TEV) coat protein were initially susceptible to TEV infection and typical systemic symptoms developed. However, 3 to 5 weeks after a TEV infection was established, transgenic plants 'recovered' from the TEV infection and new stem and leaf tissue emerged symptom- and virus-free. A TEV-resistant state was induced in the 'recovered' tissue. The resistance was virus-specific. 'Recovered' plant tissue could not be infected with TEV, but was susceptible to the closely related virus, potato virus Y (PVY). The resistance phenotype was functional at the single cell level as protoplasts from 'recovered' transgenic tissue did not support TEV replication. Surprisingly, steady state transgene mRNA levels in 'recovered' tissue were 12- to 22-fold less than transgene mRNA levels in uninoculated transgenic tissue of the same developmental age. I propose that these two observations are mediated at the cellular level by a cytoplasmic-based activity which targets specific RNA sequences for inactivation and degradation.

Introduction

The theory of pathogen-derived resistance (PDR) proposes that pathogen resistance genes can be derived from a pathogen's own genetic material (Sanford and Johnston, 1985). Numerous examples of PDR have been reported for many different plant RNA viruses in a wide range of plant species. Most examples of PDR involve transgenic plants engineered to express a viral coat protein (CP) or a segment of a replicase gene (for reviews see Beachy *et al.*, 1990; Wilson, 1993). As a general rule, transgenic plants accumulating one of these viral proteins are often resistant to that particular virus and closely related viruses. While many examples of PDR have been documented, in general the mechanism(s) underlying resistance remains to be clearly defined.

I have been examining different PDR approaches to a member of the *Potyvirus* genus. Potyviruses, assigned to the Picornaviral Super-family (for review see Goldbach, 1987), comprise a large and economically important group of aphid-transmissible plant viruses (for review see Reichmann *et al.*, 1992). Some general characteristics of potyviruses include: (1) flexuous rod-shaped virions (ca. 18 X 750nm); (2) an RNA genome that has a protein (VPg) covalently attached to the 5' terminal nucleotide and a 3' polyadenylate sequence; (3) the genetic information is contained in a single, large open reading frame on a single-stranded RNA genome (ca. 9500 nucleotides) of plus (message) sense polarity; and (4) individual gene products are expressed by proteolytic processing of the genome-derived polyprotein. Several examples of PDR to various potyviruses have been reported (for review see Lindbo *et al.*, 1993). In most cases, no correlation between transgene product accumulation and the degree of potyvirus

resistance was noted. This is in contrast to other PDR studies in other virus/transgenic plant systems where resistance correlated with high transgene expression. Thus, the exact mechanism underlying PDR for potyviruses remains enigmatic.

I have previously described the construction of a series of transgenic *Nicotiana tabacum* cv. Burley 49 lines producing different versions of the tobacco etch virus (TEV) coat protein gene sequence (Lindbo and Dougherty, 1992a). Transgenic plant lines, expressing either a full length (FL plant lines) or an amino- (N-) terminally truncated form (ΔN29 plant lines) of the TEV CP, temporally developed typical systemic symptoms when inoculated with TEV. However, FL- and ΔN29-transgenic plant lines outgrew TEV infection approximately 3 to 5 weeks after inoculation (Lindbo and Dougherty, 1992a). I refer to this phenomenon as 'recovery'. Other researchers have also noted that selected transgenic plants expressing potyvirus coat protein sequences display a similar response after inoculation with other potyviruses (Ling *et al.*, 1991; Fang and Grumet, 1993).

This study examined features of 'recovered' transgenic plant tissue. Asymptomatic 'recovered' tissue did not support TEV replication, yet supported replication of the closely related potyvirus, potato virus Y (PVY). RNA and protein analyses of 'recovered' transgenic plant tissue demonstrated that both transgene RNA and protein levels were markedly reduced in 'recovered' transgenic plant tissue. I propose that the induction of resistance to TEV, an RNA virus that replicates in the cytoplasm, and the decrease in RNA accumulation of the nuclearly transcribed transgene are mediated by a common cytoplasmically-based mechanism. Cytoplasmically-based regulation of gene expression may have important implications for (1) genetically engineered resistance to viruses, (2) classical cross protection

and naturally occurring plant virus-resistance mechanisms, and (3) transgene expression and selected examples of sense- (or co-) suppression.

Materials and Methods

Construction of transgenic plants

Construction, selection, and analyses of transgenic plants used in this study have been previously described (Lindbo and Dougherty, 1992a). The transgenic plant lines used in this study accumulate either a full length or N-terminally truncated (ΔN29) form of the TEV CP. The ΔN29 transgenic plant lines express a form of the TEV CP missing the N-terminal 29 amino acids (aa).

Virus Isolates

Tobacco etch virus strain H (TEV-H [highly aphid-transmissible] strain) was originally obtained from Dr. Tom Pirone (Kentucky). The potato virus Y (PVY) isolate used in this study was obtained from Dr. Guy Gooding (North Carolina State University). Viruses were maintained in *N. tabacum* cvs. Burley 49 or Burley 21. Virus was purified as described by Dougherty and Hiebert (1980a). Potyviral RNA was obtained from purified virus preparations by adding an equal volume of proteinase K solution (50 mM Tris-Cl, pH 8.0, 1 mM $CaCl_2$, 1% SDS, 100 μg/ml proteinase K) to virus preparations. The solution was vortexed, incubated at 45 °C for 10 min, and then extracted with an equal volume of phenol:chloroform (1:1). RNA was precipitated as described in Sambrook *et al.* (1989) and resuspended in dH_2O.

Whole plant inoculation experiments

Plant leaves were lightly dusted with carborundum and virus inoculum (50μl) was applied with a sterile cotton swab. Virus inoculum

was at a 1:10 dilution (w/v) of virus-infected plant tissue in 100 mM sodium phosphate buffer (pH 7.8).

Grafting Studies

Rootstocks were prepared by removing the shoot of the rootstock above at least two healthy basal leaves and making a vertical cut, 3 to 4 cm long, in the center of the internode. Leaves larger than 4 cm in length were removed from the scion and the base trimmed to a wedge. The cambia of stock and scion were aligned along the length of the cuts, secured with paraffin film and covered with a polyethylene bag for 7 d.

Protoplast preparation

Protoplasts were isolated from transgenic or wild-type *N. tabacum* cv. B49 leaves by lightly abrading the abaxial surface of leaves with carborundum and a cotton swab. Abraded leaf pieces were incubated overnight (15-18 h) in 1.5% cellulase "Onozuka" R-10 (Yakult Honsha Co., Tokyo, Japan), and 0.2 % macerase in mannitol-MES (0.6M mannitol, 0.1% (w/v) MES, pH 5.7). After enzyme treatment, protoplasts were floated on a 0.6 M sucrose cushion, collected and washed 2-3 times in mannitol-MES.

Protoplast transfection

The protocol of Jones *et al.*, (1990) was used except that the PEG $CaCl_2$ solution was replaced with PEG-Mg/CMS solution (1 ml 50% [w/v] PEG 1500 in 75 mM HEPES, pH 8.01 [Boehringer Mannheim] to which 15 µl 1M $MgCl_2$ and 100 µl 1M $Ca(NO_3)_2$, pH 7-9, had been added). After transfection with 2 µg of viral RNA, protoplasts were transferred to incubation media (Luciano *et al.*, 1987).

Analysis of transfected protoplasts

Protoplasts were analyzed by protoplast blotting as described by Jung *et al.* (1992). Approximately 200 viable protoplasts were pipetted onto nitrocellulose and air dried. Bound viral antigen was detected using standard immunoblotting techniques with mouse anti-TEV monoclonal antibodies, alkaline phosphatase conjugated goat anti-mouse antibodies and the chromogenic substrates NBT (para-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indoyl phosphate para-toluidine salt) (Biorad). Back inoculation of transfected protoplasts to test plants was performed as previously described (Lindbo and Dougherty, 1992b).

ELISA

The presence of viral antigen (CP) in wild-type and transgenic tissue was examined by standard double antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) procedures (Converse and Martin, 1990) using rabbit anti-TEV polyclonal sera.

Northern and Western Blot Analysis.

Northern and Western blot analysis of plant tissues was performed as previously described (Lindbo and Dougherty, 1992a, 1992b).

Aphid transmission experiments

Green peach aphids (*Myzus persicae*) were raised on mustard-spinach plants. Prior to use in transmission experiments, aphids were collected, fasted for several hours and then exposed to infected leaf tissue for 3 to 5

min. After 3 min, feeding aphids were transferred to test plant seedlings and allowed to feed overnight (8-12 hr). Aphids were then killed with an insecticide. Symptoms on test plants were allowed to develop for 10 d.

Results

Whole plant studies

All TEV-infected transgenic plant lines expressing an FL- or ΔN29-truncated form of the TEV CP recovered from TEV infection. During this process, initial infection and typical TEV-induced symptoms were succeeded by progressively less symptomatic tissue in each newly emerging leaf. In these emerging leaves, virus-induced symptoms were restricted to interveinal areas. This progressive 'recovery' continued until leaves emerged devoid of symptoms. As a result, the 'recovered' transgenic plants had a distinct appearance compared to TEV-infected untransformed Burley 49 plants (Figure V.1).

Inoculation studies with viruses other than TEV demonstrated that the recovery phenomenon was TEV-specific. These FL- and ΔN29-transgenic plant lines were susceptible to the closely related potyviruses PVY and tobacco vein mottling virus (TVMV) (Lindbo and Dougherty, 1992a), as well as a number of unrelated plant viruses (data not shown). Inoculation of these transgenic lines with these viruses established a systemic infection and no recovery was evident (data not shown). Aside from the TEV-induced 'recovery' phenotype, plant height, weight, and general morphology of transgenic plants used in this study were similar to untransformed plants.

The level of TEV in transgenic and untransformed *N. tabacum* cv. Burley 49 plants was quantified in order to determine if infectious TEV was present in tissue displaying the 'recovered' phenotype. A DAS ELISA (Converse and Martin, 1990) was used to indirectly quantitate TEV in the various tissue types. The DAS ELISA conditions used detected only CP from

Figure V.1. Tobacco etch virus (TEV)-induced symptoms in wild type and transgenic tobacco plants. TEV-induced symptoms are shown for a *Nicotiana tabacum* cv. Burley 49 plant (lower left), and for a plant from a transgenic line (FL-44.4, upper left) expressing a full-length version of the TEV coat protein. The transgenic plant has outgrown or "recovered" from TEV infection; newly emerging leaves contain less virus and virus-induced symptoms than older leaves of the same plant or than the corresponding leaf from a TEV-infected B49 plant. Leaves from the bottom, middle, or top segment (leaves 1, 2, and 3 respectively) of these plants are presented for comparison on the right. Photographs were taken approximately 5 weeks after inoculation with TEV.

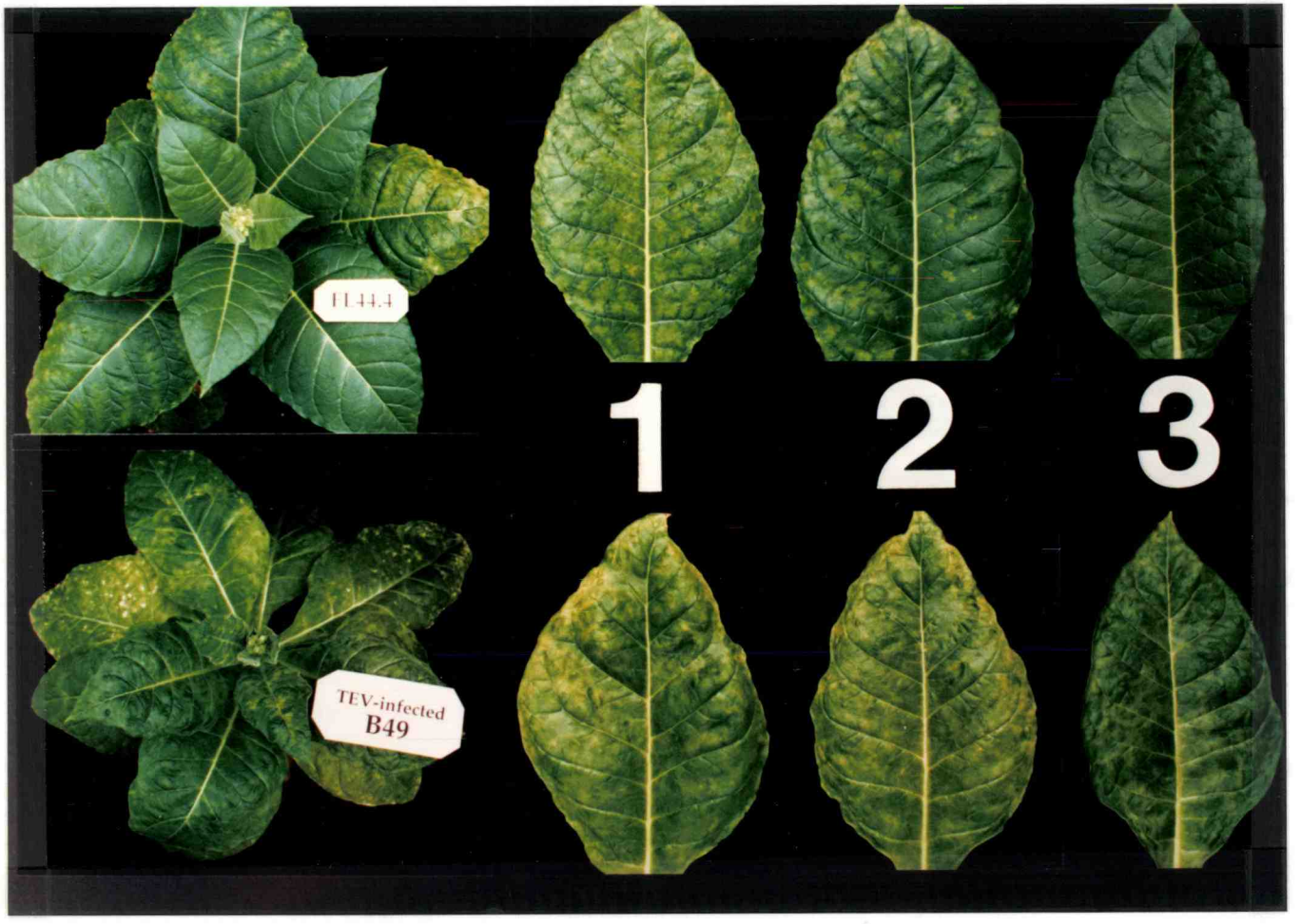


Figure V.1.

replicating TEV, not TEV CP produced from the transgene. TEV CP levels in symptomatic leaf tissue of infected transgenic plants were usually slightly lower than those in infected untransformed Burley 49 (Table V.1). However, this difference was most striking when comparing TEV CP levels of TEV-infected Burley 49 tissue to levels found in the asymptomatic tissue of plants displaying the 'recovered' phenotype. No TEV CP was detected in asymptomatic leaf tissue (Table V.1).

I attempted to demonstrate the presence of infectious TEV in transgenic tissue by bioassays involving both aphid-vectored transmission experiments and back inoculation studies to a susceptible indicator host. In general, lower TEV CP levels were correlated with lower aphid transmission rates when TEV-infected transgenic tissue was used as an inoculum source by aphids (Table V.1.). However, aphid transmission of infectious virus from asymptomatic leaf tissue of the 'recovered' phenotype or back inoculation of sap (data not shown) to *N. tabacum* cv. Burley 21 plants was unsuccessful. Therefore, by a variety of experimental approaches, TEV could not be detected in asymptomatic tissue of leaves showing the 'recovered' phenotype.

These results suggested that TEV was excluded from or could not replicate in 'recovered' transgenic tissue. Plants from the FL- and ΔN29- lines were tested for their ability to support virus replication in a sequential inoculation series. Plants from each of the lines were "pre-treated" in one of four ways prior to a challenge inoculation with TEV or PVY. The four pre-treatments were: (1) naive (receiving no initial treatment); (2) mock inoculated with buffer and carborundum on a lower leaf; (3) inoculated with tobacco mosaic virus (TMV); or (4) inoculated with TEV. No visible plant symptoms were evident in pre-treatments 1 and 2, pre-treatment 3

Table V.1. TEV concentration in TEV-infected plants

Plant line[a]	Leaf type[b]	µg virus/[c] mg tissue	Aphid transmission[e] rate
B49	1	1.45	6/6
B49	2	1.5	6/6
B49	3	1.35	5/6
FL-24.3	1	1.0	5/6
FL-24.3	2	0.4	1/6
FL-24.3	3	0.0	0/6
FL-44.4	1	1.4	3/6
FL-44.4	2	0.3	2/6
FL-44.4	3	0.0	0/6
FL-3.3	1	8.4	3/6
FL-3.3	3	0.0	0/6
N-8.1	1	1.0	3/6
N-8.1	2	0.5	1/6
N-8.1	2	0.2	2/6
N-8.1[d]	2 (symptomatic area)	5.5	3/6
N-8.1[d]	2 (asymptomatic area)	0.0	0/6

[a]Plant nomenclature used is as described in text.

[b]Leaf type 1, 2, or 3, are lower, middle or upper leaves, respectively, as shown in Fig V.1.

[c]Virus concentration was estimated using DAS ELISA. A standard curve (data not shown) was produced by adding known quantities of purified TEV to uninfected B49 extracts. Tissue extracts were analyzed by DAS ELISA procedures using alkaline phosphatase conjugated rabbit anti-TEV antibodies. Infected plant samples (except [d]) were collected by randomly removing 6 circular (10 mm dia., each) tissue samples from a leaf, combining them and then preparing tissue extracts. DAS ELISA data is presented as µg TEV/mg tissue.

[d]Symptomatic and asymptomatic tissue samples from the same 'recovering' leaf (i.e. leaf type 2, Figure V.1.) were analyzed.

[e]Aphid transmission rate indicates the number of test plants that became infected with TEV via aphid transmission over the number of test plants aphids were allowed to probe. Ten aphids were applied to each test plant.

resulted in the formation of local lesions on the inoculated lower leaf, while pre-treatment 4 resulted in systemic TEV infection followed by recovery. Four weeks after the original pre-treatment, upper leaves of these plants were challenge inoculated with either PVY or TEV. Results are summarized in Table V.2. All plants were susceptible to PVY infection. Plants receiving pre-treatments 1, 2, or 3 were infected by TEV; however, those plants which had 'recovered' from prior TEV infection were unable to support a TEV infection in the 'recovered' tissue.

Further characterization of the induced resistant state involved 'recovered' scions from various FL- and ΔN29- transgenic plants and scions from untransformed Burley 49 plants grafted on TEV-infected *N. tabacum* cv. Burley 49 rootstock. Scions from transgenic plants displaying the 'recovered' phenotype remained symptom- and virus-free, while grafted untransformed Burley 49 scions became infected and showed typical systemic TEV symptoms within 10 to 15 days after the graft was established. Resistance was absolute in the 'recovered' tissue and symptom development was never observed in the 'recovered' scions of >50 grafted plants. 'Recovered' FL- or ΔN29-scions grafted onto PVY-infected Burley 49 rootstock did become infected by PVY replication. Attempts were made to induce TEV-resistance in unchallenged FL- and ΔN29-lines and in Burley 49 tissue. Various graft combinations were established, typically by grafting untransformed Burley 49 tissue or an unchallenged FL- or ΔN29 scion onto a 'recovered' FL- or ΔN29 rootstock. In nearly all cases, the scion became infected within 10 to 20 days after grafting. This suggested that virus moved readily through 'recovered' tissue and that a translocatable signal was not involved in the induction of resistance.

Table V.2. Induction of 'recovered' phenotype in transgenic plants

Plant Line[a]	Pre-treatment[b]	Treatment	Symptoms on upper leaves[c]
Group 1 (Transgenic plant lines)	TEV	none	none
	PVY	none	systemic PVY symptoms
	TEV	TEV	none
	TEV	PVY	systemic PVY symptoms
	TMV	TEV	systemic TEV symptoms followed by recovered plant phenotype
	TMV	PVY	systemic PVY symptoms
	mock	TEV	systemic TEV symptoms followed by recovered plant phenotype
	mock	PVY	systemic PVY symptoms
	none	TEV	systemic TEV symptoms followed by recovered plant phenotype
	none	PVY	systemic PVY symptoms
Group 2 (Control plant lines)	TEV	none	systemic TEV symptoms
	PVY	none	systemic PVY symptoms
	TMV	TEV	systemic TEV symptoms
	TMV	PVY	systemic PVY symptoms
	mock	TEV	systemic TEV symptoms
	mock	PVY	systemic PVY symptoms
	none	TEV	systemic TEV symptoms
	none	PVY	systemic PVY symptoms

[a]The plant lines used in this study are presented as two groups based on their identical response to the various challenge inoculations. Group 1 plants (FL-44.4, FL-24.3, ΔN-8.1, and ΔN-1.9) displayed the 'recovery' phenotype. The nomenclature used is as described in the test. Group 2 plants were control lines that did not display the 'recovery' phenotype and were *Nicotiana tabacum* cv. Burley 49 and 35S-4.7 (a vector-only transgenic control line containing no TEV sequences).

[b]The two sequential treatments were applied 4 weeks apart. Tobacco etch virus (TEV), potato virus Y (PVY), or tobacco mosaic virus (TMV) were mechanically inoculated onto leaf tissue dusted with carborundum using a 1:10 dilution of virus infected plant sap. For the mock inoculation studies, a 1:10 dilution of uninfected Burley 49 extract was used.

[c]Symptoms were observed daily for 30 days after the second inoculation treatment.

The TEV-resistance phenotype of progeny plants from TEV-recovered ΔN29- and FL-lines were also examined. Plants showing the 'recovered' phenotype were allowed to self-pollinate and set seed. Progeny plants were grown and then inoculated with TEV-infected plant sap. These plants reacted to virus infection in a fashion identical to parental plants; plants became systemically infected and then 'recovered' from TEV infection. Therefore, progeny had not acquired the induced TEV-resistant state from their parent, but had inherited the ability to recover after TEV inoculation.

Collectively, the results obtained in whole plant studies suggested a specific anti-TEV state had been induced in the 'recovered' FL- and ΔN29-transgenic leaf tissue and TEV could not replicate in this tissue.

Protoplast studies

Studies were conducted to determine if the TEV-induced resistance was functional at the single cell level. Protoplasts from both asymptomatic tissue of TEV-infected plants displaying the 'recovered' phenotype and unchallenged FL- and ΔN29-transgenic leaf tissue were transfected with either PVY- or TEV-RNA. After incubation (ca. 24 to 48 hours), the percentage of protoplasts supporting virus replication was determined by analyzing transfected protoplasts in a protoplast printing assay (Jung *et al.*, 1992). Transfected protoplasts were also analyzed for the presence of infectious particles in back inoculation studies (Table V.3). Protoplast studies correlated with whole plant studies; protoplasts from 'recovered' tissue did

Table V.3. Analysis of TEV and PVY replication in protoplasts derived from unchallenged or 'recovered' transgenic plant tissue.

Protoplast source[a]	Percent of protoplasts supporting virus replication[b]			Recovery of infectious TEV from protoplasts infected with TEV RNA[c]
	Inoculum			
	TEV RNA	PVY RNA	NO RNA	
FL-24.3	88	59	0	+
FL-29.3	96	65	0	+
ΔN29-8.1	82	30	0	+
FL-24.3 (r)	0	57	0	-
FL-29.3 (r)	0	57	0	-
ΔN29-8.1 (r)	0	69	0	-

[a]Results from three different lines are presented. Transgenic plant nomenclature is as described in text; (r), leaf tissue with the 'recovered' plant phenotype

[b]Protoplasts were transfected with purified TEV- or PVY-RNA or with no RNA, as described in the text. Results are presented as percentage of protoplasts infected as determined in protoplast printing assays. Monoclonal anti-TEV coat protein antibodies or anti-PVY polyclonal antisera were used to detect viral antigen. Approximately 200 viable protoplasts (per sample) were counted.

[c] Protoplasts which had been transfected with TEV RNA were pelleted, lysed, and extracts were inoculated onto *Nicotiana tabacum* cv. Burley 49 leaves. Plants were examined 1 week later for the appearance of TEV-induced systemic symptoms.

not support TEV replication, while PVY replicated in protoplasts from either 'recovered' or unchallenged leaf tissue (Table V.3). Therefore, both the protoplast and whole plant studies suggested that the 'recovered' phenotype was due to the inability of TEV to replicate in 'recovered' leaf tissue.

Transgene expression

'Recovered' and unchallenged transgenic tissues were analyzed for transgene transcript and translation product accumulation. Steady state levels of transgene-encoded RNA and protein were examined by Northern and Western blot analyses, respectively. Surprisingly, transgene coat protein could not be detected in 'recovered' tissue (Figure V.2A) and transgene mRNA levels from 'recovered' tissue were greatly reduced (Figure V.2B). Quantitation of transcript levels revealed a 12- to 22-fold reduction in transgene mRNA levels in 'recovered' tissue as compared to transgene mRNA levels in unchallenged transgenic plant tissue at an equivalent stage of development. As an internal control, actin mRNA levels were also analyzed in these same RNA samples. This analysis revealed that actin mRNA levels were approximately constant in the different RNA samples (Figure V.2C).

Figure V.2. Analysis of steady state levels of transgene RNA and protein from the asymptomatic areas of 'recovered' leaves and from unchallenged transgenic plant tissue. Extracts from the same tissues are in identical lanes in **A, B** and **C**, except where noted. Protein **(A)** or RNA **(B & C)** samples were used from the following tissues: lane 1, samples from *N. tabacum* cv. Burley 49 tissue to which 10ng of purified TEV **(A)** or 100pg of an RNA transcript containing the TEV coat protein sequence was added **(B)**; lane 2: samples from *N. tabacum* cv. Burley 49 tissue; lane 3, samples from transgenic FL-24.3 plant which was not challenged with TEV; lane 4, samples from FL-24.3 transgenic plant which had recovered from TEV infection; lane 5, samples from a transgenic FL44.4 plant which was not challenged with TEV; lane 6, samples from a transgenic FL-44.4 plant which had recovered from TEV infection; lane 7, samples from a transgenic ΔN29-8.1 plant which was not challenged with TEV; lane 8, samples from a ΔN29-8.1 transgenic plant which had recovered from TEV infection.

A. Western blot analysis of transgenic and B49 plant tissue isolated from the plants described above. Total protein was extracted and electrophoresed through a 10 % acrylamide SDS-PAGE gel and then transferred to nitrocellulose membrane. The membrane was treated with rabbit anti-TEV polyclonal sera, goat anti-rabbit IgG (alkaline phosphatase conjugated) and the chromogenic substrates NBT and BCIP to detect bound TEV CP molecules. The position to which full length TEV coat protein migrates is indicated by the arrow on the left. Proteins which cross-react with our anti-TEV serum are present at the top and serve as an internal control for the amount of protein loaded in each lane.

B., C. Northern blot hybridization analyses of Burley 49 and transgenic plant tissue. Total RNA was extracted from plant tissues described above

and analyzed. RNA samples were extracted, separated by electrophoresis on denaturing (formaldehyde) agarose gel, and transferred to nitrocellulose. TEV CP transgene transcripts (**B**) or tobacco actin sequences (**C**), were detected by hybridizing filters with the appropriate ^{32}P-labeled RNA probe. Filters were exposed to X-ray film and photographs of the autoradiograms are presented. Note that no sample was loaded in lane 1 of autoradiogram in panel **C**.

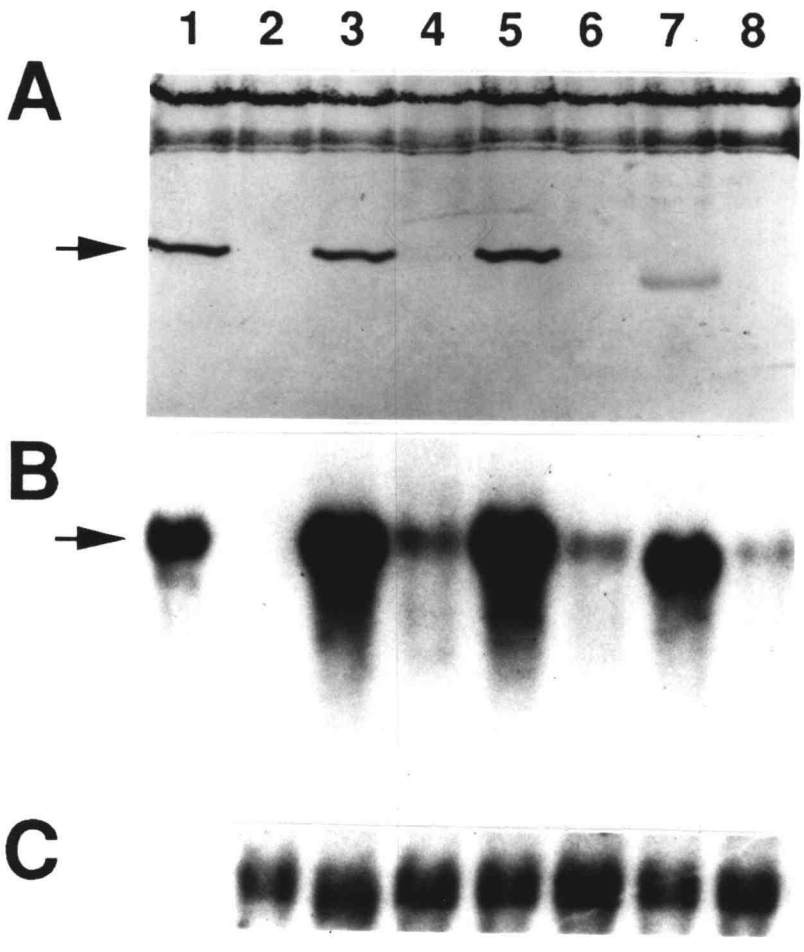


Figure V.2.

Discussion

I observed a unique, TEV-resistant state induced in transgenic plants expressing an FL- or ΔN29-terminally truncated form of the TEV CP gene. When initially challenged with TEV, these transgenic plants displayed typical TEV-induced symptoms, but gradually outgrew infection by approximately 3 weeks post inoculation. I refer to this process as 'recovery'. As a result, 'recovered' transgenic plants consist of lower, symptomatic leaves (which contain virus) and upper, asymptomatic leaves (which are virus-free). Only TEV induced this phenotype, as mock inoculation or inoculations with other viruses did not induce the 'recovered' plant phenotype. Using a variety of approaches, I examined the ability of 'recovered' tissue to support TEV replication. In whole plant studies, 'recovered' tissue could not be infected with TEV but PVY, a related potyvirus (displaying ~47% total nucleotide sequence homology; ~63% coat protein gene sequence homology with TEV), established a normal systemic infection. Comparable results were obtained in protoplast studies; protoplasts from 'recovered' tissue did not support TEV replication, but did support PVY replication.

The induced resistance observed in this report is distinct from the phenomenon of systemically acquired resistance (SAR) (for review see Kuc, 1982). SAR can be induced by a variety of pathogens after the formation of a necrotic local lesion, shows broad spectrum effects against different pathogens (viruses, fungi, and bacteria), is active in untransformed germplasm (does not require the presence of a particular transgene), and has been linked to the induction of a number of genes with the involvement of

salicylic acid as a translocatable signal (Ward *et al.*, 1991). In contrast, the induced anti-TEV state in FL- and ΔN29-plants that results from 'outgrowing' a systemic infection, is TEV-specific. The induced anti-TEV state appears to require the presence of a TEV-derived transgene. An endogenous signaling molecule does not appear to be involved, as the phenotype could not be transmitted in grafted plants. The involvement of endogenous host genes activated by viral or viral-induced signals has not been determined in our system.

Coincident with the TEV-resistant state in 'recovered' plant tissues, transgene mRNA and CP levels were significantly reduced. In 'recovered' transgenic plant tissue, no transgene CP was detected and transgene mRNA accumulation levels were reduced 12 to 22-fold compared to transgene mRNA levels of unchallenged transgenic plant tissues of the same developmental stage. These observations were surprising, as all of our transgene constructions use the enhanced cauliflower mosaic virus 35S promoter (Kay *et al.*, 1987), a regulatory element associated with constitutive transcription in transgenic plants (Odell *et al.*, 1985), though transcriptional activity may vary in certain tissues (Williamson *et al.*, 1989; Barnes, 1990). The only difference between these genetically-identical plants was whether or not the plant had been infected by TEV; therefore, I anticipated no difference in transgene expression between 'recovered' and unchallenged transgenic tissue. One explanation for my observations was that the decreased transgene expression and the induced anti-TEV state were unlinked, induced by two separate mechanisms. In this scenario, specific methylation of the transgene could be responsible for a reduction of transcription with the induced anti-TEV state mediated by another cellular process. While cognizant of this possibility, I propose that the two

characteristics common to all tested 'recovered' tissue [i.e. a decreased accumulation of a nuclear-encoded TEV mRNA transcript and the inability of TEV, an RNA virus, to replicate in the cytoplasm] are linked. I have developed a working model for our observations.

The model predicts that the molecular basis of the 'recovered' phenotype is a cytoplasmic-based event, in which RNA sequences coding for the TEV CP are specifically targeted, functionally suppressed, and eventually eliminated from the cell. I propose that this activity is directly responsible for both the decrease in transgene mRNA accumulation in 'recovered' tissue as well as the inability of 'recovered' tissue to support TEV replication. Mechanistically, the model suggests that a protein or nucleic acid factor binds to a specific RNA sequence, rendering this RNA functionally inactive and targeting it for elimination.

For the induction of the resistant state, I observed that the infecting virus (TEV) must first establish a systemic infection. This suggested that stimulation (or infection) of cells in the apical meristem was necessary. As noted previously, I have never observed TEV-infected untransformed plants to display this 'recovery' phenotype, and mock- or TMV-inoculated transgenic plants also did not attain the TEV-resistant state. Therefore, a specific interaction among TEV, the transgene, and the host plant was necessary for the induction of the resistance phenotype. What is the potential interplay between these factors?

A scenario I favor suggests that the transgene product contributes two distinct components to the 'recovery' phenomenon. I propose that the transgene transcript and the replicating TEV genome act additively to trigger a natural cellular response which down-regulates or inactivates specific RNAs in the cytoplasm. Stimulation of this system would result in a highly

resistant phenotype to TEV as TEV RNA sequences are inactivated. Normally, in an untransformed plant, the rapidly developing systemic viral infection may overwhelm this defense response before it can be established in a cell. However, in FL- and ΔN29-transgenic lines, the transgene protein product provides a second function to impede virus movement, thereby permitting cells to 'grow ahead' of the infection. This allows the anti-viral state to be established in developing tissues. Protoplast studies indicate that once established, the system functions at the single cell level.

The induction of cytoplasmic-based activity which inactivates and eliminates specific RNA sequences from a cell may explain a number of apparently disparate and unrelated phenomena. For example, I suggest that certain examples of cross protection between infectious agents, sense or co-suppression of transgenes, and the recently described examples of RNA-mediated virus resistance may be explained by such a mechanism.

In a typical demonstration of cross protection (for review see Fulton, 1986), a plant is systemically infected with a mild isolate of a particular virus. The infected plant, when challenged with a more virulent isolate of that same virus, does not support replication of the second virus. As a result, severe symptoms induced by the second virus do not develop. A host response, similar to that proposed for 'recovered' trangenic plants [i.e. enhanced viral RNA turnover], may be activated and prevent the second challenging virus from replicating. Such a mechanism would not only be functional for RNA viruses, but also could readily explain the cross protection phenomenon observed with viroids (Niblett *et al.*, 1978; Branch *et al.*, 1988).

I suggest that a cytoplasmic-based activity, similar to that proposed for our virus-induced state, may be operational for some examples of sense- (or co-) suppression. The phenomenon of co-suppression has been described for a number of different genes in transgenic plants (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; de Carvalho *et al.*, 1992; Smith *et al.*, 1990; Hart *et al.*, 1992). Attempts to overexpress a particular gene product via the introduction of a redundant transgene often results in suppression of both the endogenous gene and exogenous transgene. In a number of cases, methylation of DNA sequences correlates with reduced gene expression (Matzke *et al.*, 1989; Hobbs *et al.*, 1990; Matzke and Matzke, 1991; Hobbs *et al.*, 1993; Ottavianai *et al.*, 1993). However, in some cases, sense suppression appears to be mediated post-transcriptionally (de Carvalho *et al.*, 1992) and a cytoplasmic-based system may account for the suppression of transcript levels.

Finally, I suggest that the anti-viral state in RNA-mediated resistant transgenic plants (Lindbo and Dougherty, 1992b; de Haan *et al.*, 1992; van der Vlugt *et al.*, 1992; Pang *et al.*, 1993; Farinelli and Malnoë, 1993) and the 'recovered' CP-producing transgenic plants function by a similar mechanism. I have previously described transgenic tobacco plants containing a TEV CP gene modified by introduction of three stop codons immediately downstream of the AUG start codon (Lindbo and Dougherty, 1992b). Such transgenic plant lines express an untranslatable TEV CP mRNA. Approximately 40% of transgenic plant lines expressing this RNA are highly resistant to TEV infection, but remain susceptible to PVY, TVMV, and other viruses. The other ca. 60% of the transgenic lines express the same untranslatable TEV CP mRNA, but are sensitive to TEV as well as other plant RNA viruses. Results obtained with whole plants are reproduced at the cellular level in protoplasts. I also observed that resistance to TEV did

not correlate with transgene mRNA accumulation levels in these transgenic plants. I propose that the highly resistant plants expressing the untranslatable RNA have a cytoplasmic-based activity which functions similarly to the TEV-induced system in 'recovered' FL and ΔN29 transgenic plants. Activation of this RNA sequence-specific, cellular response renders the plants completely resistant to subsequent TEV infection, but susceptible to potyviruses with similar, yet distinct sequences. Activation of such a system may also explain why resistance and RNA accumulation levels in these plants are not correlated. Steady state levels of transgene transcripts would be a function of RNA synthesis and turnover rates. In highly resistant lines a sequence specific RNA degradation system would be activated. Transgene RNA accumulation in these lines may be lower than in susceptible transgenic lines where the system has not been activated and only synthesis is being monitored. Hence one might expect to find a negative or no correlation between RNA accumulation and resistance.

The working model proposed is far reaching in its implications and may extend to mammalian cells. I believe this experimental system, which examines specific nuclearly transcribed genes and a cytoplasmic replicating RNA virus, will permit others to elucidate the biochemical basis of resistance and this gene regulation in detail.

CHAPTER VI

SUMMARY AND CONCLUSION

Summary

Research reports of 'coat protein-mediated resistance' (CPMR) to potyviruses have described a variety of resistance phenotypes, both broad spectrum and virus specific in nature. Several mechanisms have been proposed for these resistance phenotypes including impaired virus movement and/or replication. Additionally, virus resistance has been proposed to be RNA- or protein-mediated. In most examples of potyvirus 'CPMR' no correlation between steady state transgene RNA/protein levels and virus resistance was found. As a result, a single unifying theory of potyvirus 'CPMR' has not yet emerged. It is possible that no one single mechanism of resistance does exist, and that a variety of factors (both RNA- and protein-mediated) are involved in any example of resistance. Exactly why virus resistance occurs in some, but not all, transgenic plants is not clear.

Recent work on transgenic plants which express full length (FL plants) or amino-terminally truncated forms (ΔN29 palnts) forms of the tobacco etch virus (TEV) coat protein (CP) and are able to outgrow TEV-infection has provided an interesting perspective on potyvirus 'CPMR'. In this transgenic plant system, transgenic plant tissue which has outgrown (or recovered from) TEV infection has attained an anti-TEV state and will not support TEV replication. Concurrent with this virus resistant state is the dramatic reduction

in transgene RNA and protein levels. Neither back inoculation experiments, Northern blot analysis, nor ELISAs have demonstrated the presence of infectious TEV particles, TEV RNA, or TEV protein in recovered transgenic plant tissue. Thus it appears as though recovered tissue is virus free, accumulates reduced levels of transgene RNA/protein and will not support TEV replication. In this tissue, TEV resistance is inversely correlated with steady state transgene RNA/protein levels. Recent preliminary experiments have examined the transgene transcription rates in TEV-recovered transgenic plant tissue, comparing them to transgene transcription rates in unchallenged transgenic plant tissue. Nuclear run-on analysis suggests that transgene transcription rates are roughly equivalent in these tissues. This result suggests that in TEV-recovered transgenic plant tissue, transgene RNA levels are regulated (by the host) post-transcriptionally. This regulation appears to specifically target the TEV CP RNA sequence for degradation. If this post-transcriptional regulation mechanism resides in the cytoplasm, where TEV replicates, it is easy to imagine TEV genomic RNA also being subjected to this same regulating mechanism.

All examples of potyvirus CPMR to date have focused on virus resistance phenotypes and steady state transgene RNA/protein levels. TEV-recovered transgenic plant tissue may explain why these two observations do not correlate. In TEV-recovered tissue, it appears as though RNA stability (RNA turnover rate) and <u>not</u> steady state RNA levels are <u>critical</u> for virus resistance. Since steady state RNA levels are a balance between RNA synthesis and degradation, a variety of different transgene RNA synthesis and degradation rates could lead to equivalent 'steady state' levels of RNA in different transgenic plant lines. We propose that in many examples of

potyvirus CPMR, transgene RNA degradation rates and virus resistance will be correlated; high transgene RNA degradation rates should positively correlate with virus resistance.

This transgenic plant system implicates the host plant in the virus resistant phenotype. For the most part, in discussions of potyvirus 'CPMR', the response of the host plant to the transgene and its products have been largely ignored. Steady state transgene RNA levels in TEV-recovered transgenic tissue suggests that the response of the host plant may be critical for virus resistance. It may be that under certain conditions, the host plant takes actions to downregulate or reduce the amounts of transgene encoded RNA/protein in its cells. These actions could operate at a transcriptional or post-transcriptional level. In those cases where transgene transcript is regulated post transcriptionally, by a cytoplasmically localized targetting and degradation event(s), the probability of virus resistance is increased.

Though this discussion has presented some possible factors involved in potyvirus CPMR, many questions still remain, including:

(1) Is transgene RNA stability critical for virus resistance in many or all examples of CPMR?

(2) Why or how could transgene RNA stability vary between transgenic plant lines?

(3) In those cases where transgene RNA levels appear to be regulated post-transcriptionally, what is the nature of the recognition/degradation event?

Is the transgene RNA specifically targetted for degradation? How?

(4) Are plants able to target specific self/non-self RNAs for post-transcriptional degradation? Could such a phenomenon be acting in a some examples of co-suppression or virus cross protection?

Work on these and other intriguing questions is ongoing.

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APPENDICES

APPENDIX I

AGROBACTERIUM MEDIA

I.1

AB Minimal media

AB minimal plates (500 mls)

7.5 gms Agar
2.5 gms glucose
450 mls dH_2O.

(Note: for AB minimal broth, omit agar).
Autoclave, cool to ca. 75 °C, then add:

25 mls 20X AB salts (see below).
25 mls 20X AB buffer (see below).
Cool to ca. 55 °C.
Add antibiotics (if desired.)
(Note: If adding antibiotics to minimal media, use 1/2 the concentration used on rich media plates).
Pour.

20X AB salts (1 L)

20 gms	NH_4Cl
6 gms	$MgSO_4\ 7H_2O$
3 gms	KCl
0.2 gms	$CaCl_2$
0.05 gms	$FeSO_4\ 7H_2O$

Sterilize by autoclaving.

20X AB buffer (1 L)

60 gms	K_2HPO_4 (dibasic)
20 gms	NaH_2PO_4 (mono)

Sterilize by autoclaving.

Antibiotics:

For selecting *Agrobacterium* with pCIB 542 ($Strep^r$, $Spec^r$) and pPEV (Kan^r) on minimal media use the following: 250 µg/ml Streptomycin, 25 µg/ml Spectinomycin, 25 µg/ml Kanamycin.

I.2.

MGY media

MGY media (1L)

10 g	mannitol
2 g	L-glutamic Acid (sodium salt)
0.5 g	KH_2PO_4 (monobasic)
0.2 g	NaCl
0.2 g	$MgSO_4 \cdot 7H_2O$
1.0 g	yeast extract

Add 800 mls of dH_2O and adjust pH to 7.0 (with KOH or HCl)
Add 15 gms agar and water up to volume.
Autoclave.
(NOTE: glucose can be substituted for mannitol)

Cool to 55 °C.
Add antibiotics if desired.
Pour.

I.3.

MG media

MG media

10 g	Mannitol
2 g	L-glutamic Acid (Sodium salt)
0.5 g	KH_2P0_4 (monobasic)
0.2 g	NaCl
0.2 g	$MgSO_4 \cdot 7H_2O$
1 ml	biotin (2µg/ml stock)

Add H_2O to 800 mls. Adjust pH 7.0 (with KOH or HCl)
Add 15 gms agar and autoclave.

Cool to 55 °C.
Add antibiotics if desired.
Pour.

APPENDIX II

PLANT TISSUE CULTURE MEDIA

II.1.

MS media

MS plates (1L)

4.3 gms MS basal salts (Sigma)
5 mls 200X B-5 vitamins (see below)
30 gms sucrose
800 mls dH_2O

(Note: for MS broth, omit agar).
Adjust pH to 5.7 to 5.8 with 1N and 0.1 N KOH.
Bring volume up to 1 L.
Add 7.5 gms plant tissue culture agar (Sigma).
Autoclave.

Cool to 55 °C.
Add antibiotics if desired.
Pour.

200X B-5 Vitamins:

2 gm	myo-inositol
0.2 gms	Thiamine HCl
0.02 gms	nicotinic acid
0.02 gms	pyridoxine-HCl

Dissolve above in 100 mls sterile dH_2O. Filter sterilize.
Store at 4 °C, protected from light.

II.2.

MSBN media

MSBN Agar (1L)

4.3 gms MS basal salts (Sigma)
5 mls 200X B5 vitamins (see II.1)
30 gms sucrose.
800 mls dH_2O.

Add 100 µl BAP stock to 1L of media (see below)
(final concentration = 1µg/ml).
Add 50 µl NAA stock to 1L of media (see below)
(final concentration = 0.1 µg/ml).

Adjust pH of media to 5.7 to 5.8 with 1N or 0.1 N KOH.
Bring volume up to 1L.
Add 7.5 gms plant tissue culture agar.
Autoclave.
Cool to ca. 55 °C.
Add antibiotics, if desired.
(Example: add Carbenicillin to a final concentration of 500 µg/ml and Kanamycin to a final concentration of 100-300 µg/ml).
Pour.

BAP stock solution:

Dissolve 0.01 gms 6-benzylaminopurine (BAP) in 1 ml 1N NaOH
Make up fresh for each use.

NAA stock solution:

Dissolve 0.01 gms α-naphthaleneacetic acid (NAA) in 1 ml EtOH.
Slowly add 4 mls dH_2O (while vortexing).
Make up fresh for each use.

II.3.

Protoplast incubation media

Reagents:

Stock solutions for Incubation media:

O.6 M Mannitol
109.3 gms/L

10% MES (pH 5.7),
10 gms MES/ 100 mls total volume.
Adjust pH with 1N KOH, filter sterilize.

80 mM KH_2PO_4 (pH 5.7 to 5.8)
1.088 gms per 100 mls. Adjust pH with 0.1M KOH.
Autoclave to sterilize.

400 mM KNO_3
2.02 gms per 50 mls

40 mM $MgSO_4$
0.493 gms per 50 mls

40 µM $CaCl_2$
0.294 gms per 50 mls

2 mM KI
0.0166 gms per 50 mls

20 µM $CuSO_4$
0.0005 gms per 100 mls

Incubation Media:

to 500 mls 0.6 M Mannitol add:

5 mls 10 % MES (pH 5.7)
2.5 mls 80 mM KH_2PO_4
2.5 mls 400 mM KNO_3
2.5 mls 40 mM $MgSO_4$
2.5 mls 40 µM $CaCl_2$
0.5 mls 2 mM KI
0.5 mls 20 µM $CuSO_4$

APPENDIX III

PLANT TRANSFORMATION PROTOCOLS

III.1.

Tri-parental mating

Reagents:

L-broth
L-agar plates
MGY broth (see I.2)
AB minimal broth (see I.1)
AB minimal plates (with appropriate antibiotics) (see I.1)
Nitrocellulose filters

Procedures:

Day one:

Grow up 2 ml cultures of :

(A) *E. coli*/pRK2013 in L broth containing 50 μg/ml kanamycin.

(B) *E. coli* (HB101) with wide host range plasmid (ex. pCIB200) in L-broth containing 50 μg/ml kanamycin.

(C) *Agrobacterium tumefaciens* A136/pCIB 542 in MGY broth containing 500 μg/ml Streptomycin and 50 μg/ml Spectinomycin.

Spin down 1 ml of each overnight culture and resuspend cells in 1 ml of L-broth (or MGY broth for 'C') containing no antibiotics.
Place nitrocellulose (NC) filter disc onto L-Agar plate.
Pipette 50 μl of each of the three above resuspended cultures onto the surface of NC.
Mix by pipetting up and down 5-10 times.
Let plate sit at room temperature overnight.
Do not invert plate.

Day two

Resuspend bacteria on filter with 1 ml of AB minimal broth (no glucose or antibiotics) and a P-1000 pipette.
Draw off resuspended bacteria and pipette into eppendorf tube.
Make serial dilutions of 10^{-2}, 10^{-3} and 10^{-5} and 10^{-6}.

Plate 100, 10 and 1 µl of the dilutions onto AB minimal plates containing 250 µg/ml Streptomycin, 25 µg/ml Spectinomycin and 25 µg/ml Kanamycin.

Grow cultures at room temperature. (May take several days to see colonies).

Pick isolated colonies and restreak onto AB minimal plates (containing antibiotics). Streak 5 colonies per mating for each streak purification.

Incubate plates for streak purification at room temperature.

Streak purify selected *Agrobacterium* colonies a total of 3 times before using for plant transformation.

Selected colonies can be stored as glycerol stock cultures as follows: Add 0.5 ml overnight culture to 0.5 ml sterile glycerol. Vortex to mix, and store at -70 °C.

III.2.

Leaf 'disc' transformation of *Nicotiana tabacum*

Reagents:

MGY broth (see I.2)
MS broth (see II.1)
MSBN plates (see II.2)
MSBN (Kan/Carb) plates (see II.2)
Sterile filter paper
70 % EtOH
25% Chlorox (with 2 or 3 drops of Tween 20 per 100 mls)
GA-7 vessels each containing (25-50 mls) MS agar (see II.1)

Procedures:

Preparation of leaf tissue for transformation:

Surface sterilize *Nicotiana tabacum* seeds:

Work under hood and use sterile technique.
Place 50-100 *N. tabacum* seeds in 15 ml polypropylene tube.
Add 4 mls 70% EtOH. Swirl to mix.
Let stand 90 seconds.
Remove EtOH with Pasteur pipette. (Place tip of pipette firmly against bottom of tube to avoid pulling up seeds with liquid).
Add 4 mls 25% Chlorox solution.
Let stand 10 minutes, swirling occasionally.
Remove Chlorox solution with Pasteur pipette (as described above).
Rinse seeds by adding 5 ml sterile dH_2O. Let stand 1 min, swirling occasionally.
Remove liquid with Pasteur pippette as previously described.
Repeat sterile dH_2O wash 2X.

Growing plants under sterile conditions:

Using EtOH/flame sterilized forceps and scalpel, transfer 1 or 2 surface sterilized seeds from polypropylene tube into MS agar in a GA-7 container.
Press seeds 1-2 mm below surface of agar with tip of sterile forceps.
Place lid on GA-7 and seal with parafilm.
Place under lights.
Let seeds sprout and grow under these sterile conditions.

Seeds will sprout within a week. It will be several weeks (ca 6-8) before plants will be big enough to use for leaf disc transformation.

Subculturing plants in GA-7 containers:

(Work under hood, using sterile technique)

As plants get too large for containers, remove plants, cut off all leaves and cut main stem at internodes.
Transfer one piece of stem into each new GA-7 with MS agar.
Push one cut edge of stem ca 1 cm down into agar. (Make sure the apical end of the stem is pointing upwards).
Place lid on GA-7. Seal with parafilm. Place under lights.
Stem fragments will grow leaves and roots in a ca 2 weeks.

Infection of leaf discs with Agrobacterium:

(Work under hood, using sterile technique)

Inoculate MGY broth (500 μg/ml Spec; 50 μg/ml Strep; 50 μg/ml Kan) with *Agrobacterium* colony containing suitable plant transformation plasmid.
Incubate at room temp, 200 rpm for 24-36 hours.
Dilute 24-36 hr culture 10^{-2} to 10^{-3} into 10 mls MS broth.
Let *Agrobacterium* equilibrate to MS media for ca. 4-5 hours.
Excise leaf from a plant grown under sterile conditions.
Cut leaf up into 1cm squares.
Place leaf 'discs' (pieces) into petri dish. Pipette *Agrobacterium* (in MS broth) into dish with leaf discs.
Let leaf discs sit in media for 2 minutes.
Remove leaf discs and blot onto sterilized filter paper.
Place discs onto a piece of sterile filter paper overlayed on an MSBN plate (NO antibiotics).
After three days, transfer discs directly onto MSBN with Kanamycin (100-200 μg/ml) and Carbenicillin (500 μg/ml).
Transfer leaf discs onto new MSBN Kan/Carb plates ca every 2 weeks.
Callus forms in ~ 10 days to 2 weeks. Subculture callus as needed (every two weeks or so).
As shoots form, excise them from the callus and place in MS plates containing 100-200 μg/ml Kanamycin. Gently place cut end of shoot into agar.
In about 1 week roots will begin to form on shoots.
About 2 weeks after rooting begins, plantlets can be transferred to soil.
Carefully remove all agar from roots with a gentle spray of water.
Transplant into soil and immediately transfer to greenhouse (no need to "harden off").

III.3.

Selection of Kan ^{r} transgenic tobacco seedlings

Reagents:

Sterile dH_2O
70 % EtOH
25 % Chlorox with 2 or 3 drops of Tween-20 per 100 mls.

Seed Screening plates with Kan (per liter):

4.3 gms MS salts (Sigma).
Add 800 mls dH_2O.
Adjust pH to 5.7 to 5.8 (with 1N or 0.1N KOH).
Bring vol up to 1L.
Add 7.5 gms agar.

Autoclave. Cool to about 55 °C.
Add 125 µg Kanamycin Sulfate per ml.
Pour plates.

Procedures:

Surface sterilizing seeds (See III.2 for details).

Soak in 70% EtOH, 90 seconds.
Soak in 25% chlorox solution for 10 minutes.
Wash 3X in sterile dH_2O.

Selection of Kan^{r} seedlings:

Place seeds onto Seed Screening plates containing 125 ug/ml Kanamycin (Kan).
After about 5 days seeds begin to germinate.
Kan sensitive plants will develop seed leaves, but not true leaves.
Kan sensitive plants will turn white and can usually be identified after 3 weeks.

APPENDIX IV

PROTEIN PROTOCOLS

IV.1.

10 % SDS-PAGE minigel

Reagents:

10 % SDS (sterile)
1.0 M Tris (pH 8.8)
1.25 M Tris-Cl (pH 6.8)
10% (w/vol) Ammonium persulfate (made fresh)
30% Acrylamide, 0.8% Bis-acrylamide
TEMED

Laemmli Running buffer (1L, 10X):

30.25 gms	Tris
144 gms	Glycine
10 gms	SDS

Disolve in H_2O, bring volume to 1L

Procedures:

10% Polyacrylamide (discontinuous) gel.

In two small beakers, mix all ingredients for Running and Stacking gel except TEMED and Ammonium persulfate.

Running Gel		Stacking Gel	
0.15 mls	10% SDS	0.05 mls	10% SDS
5 mls	Acrylamide	0.9 mls	Acryl. mix
5.6 mls	1.0 M Tris ph 8.8	0.5 mls	Tris-Cl (pH 6.8)
4.125 mls	water	3.45 mls	water
5 µl	TEMED	5 µl	TEMED
100 µl	Amm Pers. (10% w/v)	50 µl	Amm pers. (10% w/v)

Add TEMED and ammonium persulfate to running gel solution.
Swirl to mix.
Pour running gel into assembled plates. Overlay with dH_2O.
After gel has polymerized pour off dH_2O, rinse with dH_2O.
Dry inside of plates with filter paper.

Add TEMED and ammonium persulfate to stacking gel. Swirl to mix.
Immediately pour stacking gel and insert comb.
Run gel with 1X Laemmli running buffer.
Gel should be run at about 75 to 100 mAmps.

Notes: Run gel until bromophenol blue marker in sample (see dissociation solution section IV.2) is almost off the gel (ca 3 hours) or until desired.
This recipe is enough for 2 SDS-PAGE minigels.

IV.2

Western blot analysis

Reagents:

Towbin Transfer buffer (per liter)

3.03 gms	Tris
14.41 gms	Glycine
200 mls	Methanol
800 mls	dH_2O

Dissolve Tris and glycine in water first, then add methanol.
Store at 4 °C.

TBS (per liter):

50 mls	1M Tris-Cl (pH 7.5)
11.6 gm	NaCl
1000 mls	dH_2O

TTBS (per liter):

TBS with 1 ml of Tween-20 per L.

Carbonate buffer (1L):

8.4 g	$NaHCO_3$
0.2033 g	$MgCl_2 . 6H_2O$

Dissolve in 800 mls H_2O
Adjust pH to 9.8 with NaOH
Bring volume to 1L. Autoclave.

NBT stock solution:

30 mg	p-nitro blue tetrazolium chloride
0.7 mls	N,N-dimethylformamide (DMF)
0.3 mls	dH_2O

Store at 4 °C, in dark.

BCIP stock solution:

15 mg	5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt
1 ml	DMF

Store at 4 °C, in dark.

Laemmli Running buffer (1L, 10X):

30.25 gms	Tris
144 gms	Glycine
10 gms	SDS

Disolve in H_2O, bring volume to 1L

3X dissociation solution (100 mls):

1.125 g	Tris-HCl
3 g	SDS
15 mls	glycerol
15 mls	2-mercaptoethanol
0.25 gms	Bromophenol blue

Dissolve Tris in 50 mls dH_2O. Adjust pH to 6.8.
Bring volume to 70 mls and add other ingredients.

Procedures:

Extraction of proteins:

Grind tissue in 10 vols (w/vol) of 2X Laemmli running buffer.
Clarify extracts with 10 min spin at 12.5 K in microfuge.
Remove supernatant into new tube and save.
Store supernatant (extract) at -20 °C.

Electrophoresis of proteins.

Add 5 µl of 3X dissoc. solution to 10 µl extract.
Heat in boiling water bath 3 min. Spin down condensate.
Load all 15 µl onto gel (10 % acrylamide SDS-PAGE).
Run minigel at 75 mA constant current until dye front is within 1 cm of bottom (ca. 3-4 hours).

Transfer of proteins to nitrocellulose

Assemble transfer apparatus, as follows:

Materials:

2 pieces of filter paper and 1 piece of nitrocellulose (NC), each slightly larger than gel.

Note: Always wear gloves when handling NC.

Prewet filter paper and nitrocellulose in Towbin buffer.
Place one piece of filter paper on sponge pad.
Place gel on top of this piece of filter paper.
Place NC membrane on top of gel. Make sure no bubbles are under NC.
Place second piece of filter paper on top of NC.
Place another sponge pad on top of filter creating a "gel sandwich".
Place in plastic frame of blotting apparatus.
Place frame (containing gel) into transfer apparatus filled with cold (4°C Towbin buffer).
Transfer proteins to nitrocellulose for ca. 45 min at 0.5 to 1.0 Amps. Remember, run to red. Proteins will migrate to red (+) pole. Make sure the nitrocellulose is in between gel and (+) pole.
After transfer is complete, disassemble "gel sandwich".
Gently remove NC and place in seal-a-meal bag.

Blocking of NC:

Block NC by adding 3% gelatin in TBS* (ca 15 mls solution per 60 cm^2 of NC) to seal-a-meal bag containing NC.
Block at room temp for 2 hours (minimum).

Binding primary antibody:

Pour out blocking solution from bag.
Add primary antibody diluted in 1% gelatin in TTBS*.
(For detecting TEV CP use: 1:4000 of rabbit anti-TEV). Use same volume of solution as used for blocking.
Bind primary antibody at room temperature for 4 hours to overnight.

Washing NC:

Remove NC from bag.
Place NC in dish or other suitable container.
Wash by adding enough TTBS so that NC is covered by a layer of TTBS at least 2 mm thick.
Place on shaker (50 rpm) for 7 minutes.
Pour off TTBS.
Repeat washing with TTBS 2X.

Binding secondary antibody:

Place NC into seal-a-meal bag.

Add second antibody, diluted in 1% gelatin in TTBS*.
(Ex; 1:1000 of Goat anti-rabbit Alkaline Phosphatase.) Use same volume of solution as you used to block NC.
Bind second antibody 1-2 hours at room temp.
Remove NC from bag and place in suitably sized container.
Wash (as described above) in 3X in TTBS then 1X in TBS (7 min per wash).

Color Reaction:

Place NC in new seal-a-meal bag. Add substrate solution and seal bag.
Use a volume of substrate solution (ca. 15 mls per 60 cm^2).
Substrate solution:
Add 100 µl of BCIP and 100 µl of NBT stock to 10 mls carbonate buffer.
Let color reaction develop at room temperature.
Stop reaction by removing NC from bag and rinsing blots in a dish of distilled water.
Let blots air dry on filter paper. Store in seal-a-meal bag.

*Note: To dissolve gelatin in TBS/TTBS you will need to heat solutions briefly in microwave. Let cool to room temp before using.

IV.3

Enzyme-linked immunosorbent assay (ELISA)

Reagents:

5X PBS (2 L)

80 gms	NaCl
11.5 gms	$Na_2HPO_4 \cdot 2H_2O$
2.0 gms	KH_2PO_4
2.0 gms	KCl
2.0 gms	NaN_3

Disolve in 1900 mls dH_2O. Adjust pH to 7.4.
Bring volume to 2L
Store at room temp.

Coating buffer (1L)

1.59 gms	Na_2CO_3
2.93 gms	$NaHCO_3$
0.2 gms	NaN_3

Add 990 mls of dH_2O. Adjust pH to 9.6.
Bring volume to 1L. Store at 4 °C.

Washing buffer (1L):

200 mls	5X PBS
800 mls	dH_2O
1 ml	Tween 20

Store at room temperature.

Virus buffer (1 L):

200 mls	5X PBS
700 mls	dH_2O
20 gms	polyvinyl pyrrolidone
2 gms	ovalbumin
1 ml	Tween-20

Mix ingredients. Adjust pH to 7.4. Bring volume to 1L.
If precipitate forms, filter through cheesecloth.
Store at 4 °C.

Substrate buffer (1L):

97 mls	Diethenolamine
800 mls	dH_2O
0.2 gms	NaN_3

Mix ingredients, adjust pH to 9.8. Bring volume to 1L. Store at 4 °C.

para-nitrophenyl phosphate (substrate) tablets (Sigma).
Falcon brand ELISA plates
Alkaline phosphatase conjugated antibody (anti-TEV alkaline phosphatase)
Partially purified anti-TEV IgG (coating antibody)

Procedures:

Coating ELISA plate:

Pipette 200 µl per well of rabbit anti-TEV IgG (coating antibody), diluted 1:1000 in coating buffer.
Wrap plates tightly in saran wrap.
Place at 37 °C for 4 hours or at 4 °C overnight.
Pour out anti-sera from wells.
Fill wells with wash buffer. Dump out and re-fill.
Let plate stand 3 minutes then dump out. Tap (pound) wells dry on paper towels. Make sure no liquid remains in wells.
Repeat this 3 min wash step 3 times, drying plate between washings.

Sample preparation and binding:

Collect tissue samples of interest.
In mortar and pestle, grind 0.5 gms tissue in 5 mls virus buffer.
Pour homogenate over glass wool into centrifuge tubes.
Dilute plant sap sample 1:100 in virus buffer.
Add sample dilutions (200 µl per well) to seperate wells in the ELISA plate.
Wrap in plastic wrap.
Incubate at 4 hours at 37 °C or overnight at 4 °C.
Wash and pound dry as described above (3 washes of 3 minutes each, 3 times).

Binding conjugate antibody:

Add 200 µl per well of conjugate antibody diluted 1:1000 in coating buffer.
Wrap in plastic wrap.
Incubate 4 hours at 37 °C, or overnight at 4 °C.
Wash and pound dry as previously descibed (3 washes total).

Color generation:

Add para-nitrophenyl phosphate (PNP) to substrate buffer to give a final [] of 1 mg/ml PNP.
Add 200 µl PNP solution per well.
Place plate in dark at room temp.
Read plates at 1 hour. May be necessary to let plates sit (in dark) for several hours and then read again.

Note: This particular ELISA method is a double antibody sandwich (DAS) method.

IV.4

In vitro translation: rabbit reticulocyte system

Reagents:

Translation kit stock solutions (see below)
Rabbit reticulocyte lysate
(purchased from Green Hectares, Oregon, Wisconsin).
Translatable RNA

Procedures:

Pretreatment of lysate (to remove endogenous mRNA):

		[Final]
183.5 µl	lysate	--
4.0 µl	1.0 M Tris-HCl (pH 8.2)	20 mM
2.0 µl	Creatine kinase (5 mg/ml)	50 µg/ml
2.0 µl	0.1 M $CaCl_2$	1 mM
1.5 µl	4mM Hemin	30 µM
1.0 µl	2.0 M KOAc	10 mM
2.0 µl	Micrococcal nuclease (1mg/ml)	10 µg/ml

Incubate 10 min at 30 °C.
Add:

4.0 µl	0.1 M EGTA	2 mM
18 µl	2.0 M KOAc	100 mM
12.5 µl	0.5 M Creatine Phosphate	17.4 µM
12.0 µl	tRNA (3 mg/ml)	3 µg
7.2 µl	Amino acids (- Met)	100 µM
2.5 µl	0.7 M DTT	5 mM
3.0 µl	RNasin (Promega)	
30 µl	12 mM MgOAc	1 mM
Var.	^{35}S Met (6 µl for 12 rxns)	2-5 µCi.
Var.	dH_2O	
300 µl	Total volume (treated rabbit reticulocyte lysate)	

In vitro Translation Reaction:

25 µl treated rabbit reticulocyte lysate
5 µl RNA sample

Incubate at 37 °C for 60 minutes.

Analysis of translation products:

Remove 10 µl of translation reaction.
Add 5 µl 3X dissociation solution (see **Reagents**, IV.1).
Heat in boiling water bath, 3 min.
Load all 15 µl onto SDS PAGE gel and separate proteins by electrophoresis.

APPENDIX V

RNA METHODS

V.1.

In vitro transcription using T7 or SP6 RNA polymerase

Reagents:

Restriction enzyme
Restriction enzyme buffer
5X Transcription buffer (Promega)
100 mM DTT
4 mM (each) rNTPs (ATP, GTP, UTP, CTP)
RNasin (Promega)
SP6 or T7 RNA polymerase (Promega)
RNase free DNase (0ptional), (Promega RQ1 DNase)

Procedures:

Linearize DNA:

Use a restriction enzyme which leaves a 5' overhang or a blunt end.

Example:

5 µg of DNA (or 15 µl of miniprep DNA).
5 µl of 10 restriction enzyme buffer
3 µl enzyme
water up to 50 µl

Digest at 37 °C for ca. 1.5 hours.
Remove 1 µl for analysis on a gel. If DNA has been cut completely procede to next step. If not, continue digestion.
Phenol extract (1X) then ether extract (2X).
Precipitate DNA by adding:

20 µl 5M NH_4OAc (i.e. 0.4 volumes)
140 µl 95 % EtOH (i.e. 2 volumes)
Invert to mix.

Place at -20 °C about 1 hour. Spin down (12.5 K, 10 min in microfuge).
Pour off supernatant (save pellet).
Wash by adding 0.5 ml 70% EtOH. Spin 12K, 1 min.
Pour off supernatant.
Air dry pellet, then vacuum dry.
Resuspend pellet in 20 µl dH_2O.
Store at -20 °C until needed.

Transcription Reaction:

6 µl 5X buffer
6 µl NTPs (4 mM, each, of ATP, CTP, UTP, GTP)
3 µl DTT
0.5 µl RNasin
0.7 µl T7 or SP6 RNA polymerase
14 µl linearized DNA sample

Mix with pipette and place at 37 °C for 1 hour.
Optional: If for some reason you wish to degrade the input plasmid DNA, you can now add 1 unit of RNase free DNase per µg of DNA and let digest at 37 °C for 15-30 minutes.
Remove 1 µl and run on gel.
Immediately extract remainder of reaction with an equal volume of phenol:$CHCl_3$.
Remove aqueous phase and place in clean tube.
To each of 30 µl of aqueous recovered add:
60 µl dH_2O
60 µl 5M NH_4OAc
400 µl EtOH
Invert to mix.

Store at -20 °C (30-40 min minimum).
Spin down in microfuge at 12.5 K for 5 min. Pour off supernatant.
Wash pellet by adding 0.5 ml 70% EtOH. Spin 12K, 1 min.
Pour off supernatant, save pellet.
Air dry pellet, then vacuum dry.
Resuspend pellet in 25 µl H_20. Store at -20 °C until needed.

V.2.

RNA extraction from plant tissue

Reagents:

RNA Ext. Buffer:
- 0.1 M LiCl
- 0.01 M EDTA
- 1% SDS
- 0.1M Tris-Cl pH9.0
- (Sterilize by Autoclaving)

4M LiCl (sterilize by autoclaving)
Sterile 1.5 ml Eppendorf tubes
Ceramic mortar and pestle.
Liquid N_2.
Phenol : CHl_3 (1:1)
5M NH_4OAc
EtOH (95% and 70%)

Procedures:

In a mortar and pestle, grind piece of tissue (about the size of a 50 cent piece) to a fine powder in the presence of liquid nitrogen.
Add 2.5 mls of RNA extraction buffer to powdered tissue and homogenize tissue.
Remove about 2 mls of extract and pipette equally into 3 (1.5ml) tubes each containing 600 µl of (1:1) Phenol: $CHCl_3$.
Vortex for 30 seconds to 1 minute.
Spin tubes at 12.5 K for 10 minutes in microfuge.
Remove a total of two 700 µl aliquots (of aqueous layer) from the 3 tubes and add to an equal vol. of 4M LiCl in 2 1.5 ml tubes.
Let RNA ppt. 4 hours to overnight on ice.
Spin down RNA at 12.5 K for 10 minutes in microfuge.
Pour off supernatant, save pellet.
Resuspend pellet in 100 to 200 µl dH_2O.
Add 0.4 volumes of 5M NH_4OAc and 2.5 volumes of cold 95% EtOH.
Invert to mix.
Precipitate RNA at -20°C for 2-4 hours. Spin down RNA with 12.5 K 10 min spin in microfuge. Pour off supernatant.
Wash pellet by adding 0.5 ml cold 70% EtOH. Spin 12 K, 1 min.
Pour off supernatant.
Air dry pellet, then vacuum dry.

Resuspend pellet in 10-20 µl dH_2O.
Quantitate RNA with OD readings:
    Make 500 µl of a 1:500 dilution of RNA.
    Record OD 260 and OD 280.
    (1 OD 260 = 40 µg/ml RNA.)

Final concentration of RNA is usually around 1-5 mg/ml.

V.3.

RNA dot/slot blot protocol

Reagents:

20 X SSC (see V.5)
Formaldehyde
Nitrocellulose
3mm filter paper

Procedures:

RNA sample preparations (to denature RNA)

Mix reagents as follows:

Formaldehyde	RNA	10X SSC
5 µl	var.	to 30 µl final volume

Heat at 65 °C for 10 minutes.
Ice 10 minutes.
Add 30 µl 10X SSC.

Assembly of blotting apparatus:

Cut out one piece (each) of Whatman 3mm paper and nitrocellulose to fit blotting apparatus.
Prewet filter paper and nitrocellulose in dH_2O,then soak in 10X SSC (15 min.).
Assemble apparatus with nitrocellose on top of filter paper.

Binding RNA to nitrocellulose:

Add denatured RNA samples to assembled apparatus.
Pull through apparatus with vacuum pump.
Remove filter from apparatus.
Place between Whatman 3mm paper to air dry.
Bake 30 min at 70 °C in vacuum oven.

V.4.

Denaturing (formaldehyde) RNA gels

Reagents:

10X FF Buffer
200 mM HEPES (pH 7.8)
10 mM EDTA

Deionized Formamide
Deionize with Amberlite beads: Add about 250 µl of Amberlite beads to ca. 1 ml of formamide in a 1.5 ml tube. Vortex to mix. Let sit about 15 min before using. Store in dark at room temp.

Formaldehyde (36%, reagent grade)
Agarose (electrophoresis grade)

Procedures:

Clean electrophoresis chamber, gel tray and gel comb:

Fill gel chamber (containing gel tray and comb) with 10% SDS solution.
Let soak ca. 10 minutes. Rinse with sterile dH_2O. Air dry or use clean chem-wipes to dry.

Pour denaturing RNA gel

1.2 % Agarose gel (6% formaldehyde):

0.3 gm Agarose
2.5 ml 10X FF buffer
18.5 mls dH_2O
Dissolve agarose by heating in microwave.
Cool to 55 °C. Then Add:

4.2 mls Formaldehyde.
(25 mls Total Vol.)

Pour gel (under hood) immediately after adding formaldehyde.
Let gel set and cool under hood.

RNA Sample Prep (15 µl or 10 µl final volume):

RNA Sample	Formamide	Formaldehyde	H_2O	10X FF buf.	Vol.
3.5 µl (max)	7.5 µl	2.5 µl	x µl	1.5 ul	15 µl
2.3 ul (max)	5 µl	1.6 µl	x µl	1 µl	10 µl

Mix all components together.
Heat to 65 °C for 10 minutes.
Ice for 10 minutes.
Mix and load onto gel.
Add bromophenol blue (BpB) to (at least) one lane for marker.
Run gel at 10-20 mA until BpB is near bottom of gel.
Transfer to Nitrocellulose.

V.5.

Transfer of RNA to nitrocellulose.

Reagents:

20 X SSC
175.32 g NaCl
88.23 g NaCitrate.
volume to 1L
Autoclave

Nitrocellulose
Whatman 3mm filter paper
Plastic (Saran) wrap

Procedures:

Set up:

Cut 10 pieces of Whatmann 3mm filter paper, and one piece of nitrocellulose (NC), each slightly larger (within a cm or 2) than gel.
Soak NC in dH_2O for 5 min., then in 20X SSC for 5 minutes.

Transfer:

On a piece of plastic wrap place 7 pieces of Whatman 3MM paper.
Saturate filter paper with 20 X SSC.
Place RNA gel on top of whatman paper.
Fold plastic wrap up to rest on the top of the gel on all 4 sides. This "seals" the edges of the gel, on all sides.
Place pre-wetted nitrocellulose membrane on top of gel.
Remove all air bubbles by using a 5 or 10 ml pipette like a "rolling pin".
Saturate remaining 3 pieces of filter paper with 20 X SSC. Place on top of NC.
Place a stack of paper towels (about 5 inches high) on top of filter paper.
Place a weight (about 500 gms) on top of towels.
Let transfer overnight.

Binding of RNA to Nitrocellulose (NC):
After transferring overnight, remove NC and place between 2 pieces of dry 3mm filter paper.
Let NC air dry.
Bake at 70 °C in vacuum oven for 30 min to 2 hours.

V.6.

Hybridization of nitrocellulose filters

Reagents:

Pre-hyb buffer (500 mls):
87.66 gms NaCl
44.12 gms NaCitrate
2.0 gms Ficoll
2.0 gms PVP
Volume to 500 mls with dH_2O. Autoclave. Cool, then add
2.0 gms BSA
(alternatively can add all ingredients and filter sterilize).

1 M $NaPO_4$ (pH 6.5)
Denatured Salmon Sperm (10 mg/ml)
10 % SDS
Formamide
20X SSC (see V.5)

Procedures:

Pre-hybridize:

Incubate baked filter for at least 2 hours (55-65° C) in a seal-a-meal bag containing pre-hyb solution. Use about 20 mls pre-hyb solution per 150 cm^2 of nitrocellulose (NC).

Pre-hyb solution:
5 mls Pre-hyb buffer
0.4 mls $NaPO_4$ (pH 6.5)
0.5 mls SS DNA (10 mg/ml) (boil 5 min before adding).
2.0 mls 10% SDS
2.1 mls dH_20
10 mls Formamide.
(Total vol = 20 mls.)

Hybridization

Cut open bag. Add RNA (ribo-) probe to pre-hyb solution and reseal bag.
Hybridize at 65 °C for at least 4 hours.

Wash conditions:

Cut corner of bag and drain out probe into suitable container (ex a 50 ml screw top plastic tube). Probe can be re-used several (2-3) times, if stored at -20 °C.
Gently cut bag to release filter. Gently place filter in dish.
Wash filter by adding enough 2X SSC to cover NC filter with a layer of liquid at least a couple of mm thick.
Wash (50 rpm on shaker) at room temp. for 15 minutes. Pour out 2X SSC and repeat wash with fresh 2X SSC.
Pour out 2X SSC and wash filter in 0.1X SSC, on shaker (50 rpm), at 60-65 °C for 1 hour.
Repeat 0.1X SSC wash.
Remove NC from final wash. Place on dry 3mm filter paper.
Air dry filter. Wrap in saran wrap and expose to X-ray film.

V.7.

Synthesis of riboprobe

Reagents:

5X transcription buffer (Promega)
100 mM DTT
RNasin (Promega)
ATP, GTP, UTP mix (2.5 mM each)
100 µM CTP (cold)
T7 or SP6 RNA polymerase (Promega)
Linearized DNA template
α-^{32}P CTP (800 or 3000 µCi/mmole)

Procedures:

Probe synthesis:

Add Reagents in the following order:

4 µl	5X buffer
2 µl	DTT (100 mM)
0.5 µl	RNasin
4 µl	2.5 mM ATP, GTP, UTP mix
2.4 µl	100 µM CTP (cold)
1.0 µg	Linearized DNA template
5.0 µl	(α–^{32}P) CTP (50 µCi at 10mCi/ml)
1.0 µl	T7 or SP6 Polymerase
var.	dH_2O
20 µl final vol.	

Mix all ingredients briefly.
Let reaction procede at 37 °C for 1 hour.
Add 1 unit RNase free DNase (to destroy template).
Incubate 15 minutes at 37 °C.
Add (8 µl) NH_4OAc and (70 µl) EtOH to precipitate RNA.
Place at -20 °C (ca. 2 hours).
Spin down RNA (12.5 K, 10 minutes in microfuge).
Remove supernatant with a pipette.
Resuspend RNA in (50 µl) TE or dH_2O.
Add directly to heated pre-hyb mix in a seal-a-meal bag already containing nitrocellulose filter.

APPENDIX VI

DNA METHODS

VI.1.

Oligonucleotide purification: ethanol precipitation method

Reagents:

5M NH_4OAc
EtOH (95% and 70%)
TE (10 mM Tris pH 8.0, 1 mM EDTA)

Procedures:

Resuspend 0.2 µmoles of lyophilized oligo in 1 ml TE.
Remove 200 µl. Store remaining 800 µl in freezer (-20°C).
To 200 µl sample add:

200 µl 5 M NH_4OAc
800 µl 95% EtOH
Invert to mix.

Let sit at -20 °C for 15 minutes.
Spin 5 minutes at 12 K.
Pour off supernatant.
Air dry pellet.
Resuspend in 200 µl TE. Add:

200 µl 5 M NH_4OAc
800 µl 95% EtOH
Invert to mix.

Let sit at -20 °C for 15 minutes.
Spin down for 5 minutes at 12K.
Pour off supernatant. SAVE PELLET.
Wash by adding 0.5 ml cold 70% EtOH. Spin 12K, 1 min.
Pour off supernatant. Save pellet.
Air dry pellet, then vacuum dry.
Resuspend pellet in 200 µl TE.
Record OD 260 and 280 of 500 µl of a 1:100 dilution.
Use 1 OD 260 = 20 µg/ml oligonucleotide.

VI.2.

Phosphorylation of oligonucleotides

Reagents:

10X kinase buffer
- 0.5 M Tris-Cl (pH 7.6)
- 0.1 M $MgCl_2$
- 50 mM DTT
- 1 mM spermidine
- 1 mM EDTA

100 mM ATP
T4 polynucleotide kinase

Procedures:

Mix the following reagents together in an eppendorf tube.

- 50 picomoles of oligonucleotide
- 3 µl of 10X Kinase buffer
- 0.5 µl of 100 mM ATP
- 1 µl of T4 polynucleotide kinase
- Sterile H_2O to 30 µl

Incubate at 37 °C for 30 minutes.
Heat kill kinase at 70 °C for 10 minutes.
Final oligo [] is 1.6 pmoles/µl.
Phosphorylated oligo can be used directly for site directed mutagenesis (Amersham kit).

VI.3.

Plasmid DNA miniprep (boiling method)

Reagents:

STET:
8% Sucrose
0.5 % Triton X-100
50 mM EDTA (pH 8.0)
10 mM Tris-Cl (pH 8.0)
Autoclave.

Lysozyme:
10 mg/ml in 10 mM Tris-Cl pH 8.0
Store stock at -20 °C.

L-broth (1L):
5 gms NaCl
5 gms Yeast extract
10 gms Tryptone
Autoclave.

Sterile 1.5 ml Eppendorf tubes
Phenol:$CHCl_3$ (1:1)
$CHCl_3$
5 M NH_4OAc
EtOH (95% and 70%)

Procedures:

In large culture tube (ca 40 ml) inoculate ca. 5 mls of L-broth (containing the appropriate antibiotics) with *E. coli.*
Culture at 37 °C, 250-300 rpm overnight.
Transfer ca. 1 ml broth culture into 1.5 ml Eppendorf tube.
Spin 4 min at 9K in Tomy microfuge.
Remove supernatant by aspiration.
Resuspend pellet in 325 ul STET.(Vortex hard).
Add 15 µl lysozyme stock (10 mg/ml)
Let stand at room temp. for 5 min.
Put in boiling water bath for 1 min.
Place tubes on ice until they cool at least to room temp.
Spin 10 min. at high in microfuge.
Vortex hard.
Spin 10 min at high.

Remove pellet with a toothpick and throw pellet into the biohazard waste.
Add 1 µl RNase to super and let sit 15 min. at room temp.
Extract with Phenol: $CHCl_3$ (1:1) 2x.
Extract with $CHCl_3$ 1x. Place aqueous layer in clean tube and add:
125 µl 5M NH_4OAc.
900 µl EtOH
Invert to mix.
Place at - 70° C for 30 min.
Spin 6 min at high in microfuge.
Pour off super.
Wash pellet with 0.5 ml 70 % EtOH. Spin 3 min at high in microfuge.
Pour off super. Air dry, then dry in speed vac.
Resuspend in 50µl dH_20.
Run 2 µl on gel to analyze.

VI.4.

Extraction of DNA from plant tissue

Reagents:

2X CTAB buffer (Autoclaved)

2%	CTAB (w/v)
100 mM	Tris (pH 8.0)
20 mM	EDTA (pH 8.0)
1.4 M	NaCl
1 %	PVP (polyvinylpyrrolidone) MW 40,000

5% CTAB solution (Autoclaved)

5 %	CTAB
0.35 M	NaCl

CTAB Precipitation buffer (Autoclaved)

1%	CTAB
50 mM	Tris (pH 8.0)
10 mM	EDTA (pH 8.0)

****Note:** CTAB = Hexadecyltri-methylammonium bromide**

High-salt TE buffer (Autoclaved)

10 mM	Tris (pH 8.0)
1 mM	EDTA (pH 8.0)
1M	NaCl

0.1 X TE buffer (Autoclaved)

1 mM	Tris (pH 8.0)
0.1 mM	EDTA (pH 8.0)

RNase stock solution:

1 mg/ml	RNase A
100 U/ml	RNase T1

Heat in boiling water bath for at least 10 minutes (to destroy any DNases.)

Store stock solution at -20 °C.

$CHCl_3$
Liquid N_2
Mortar and Pestle
Sterile 1.5 ml Eppendorf tubes
EtOH (95% and 70%)
5M NH_4OAc

Procedures:

Prewarm 2X CTAB buffer and 5% CTAB solution in 65 °C H_2O bath.
Also dilute some 2X CTAB buffer to 1X (ca. 1 ml of 1X CTAB buffer per gm tissue to be extracted should be enough). Place 1X CTAB buffer in 65 °C water bath too.
Weigh tissue.
Grind to powder in the presence of liquid nitrogen.
Add 1 vol (i.e. 1 ml buffer per gram tissue) 2X CTAB buffer (65 °C). (Should be like thick soup. If too dry add 1X CTAB buffer).
Transfer slurry to eppendorf tube (≈ 0.5 mls per tube).
Add equal vol chloroform. Vortex to mix.
Spin 30 seconds at 11K. If aq. vol is not as large as $CHCl_3$, add 1X CTAB buffer to bring up volume.
Transfer aq layer into new tube. Add 1/5 vol of 5% CTAB. (eg. to 600 µl aq add 120 µl 5% CTAB)
Extract with $CHCl_3$. Spin 30 seconds at 11K.
Remove aq layer to new tube. Add equal vol of CTAB ppt buffer. Invert gently to mix.
Spin 60 seconds at 11K. Pour off supernatant.
Resuspend pellet in high salt TE (ca. 200 µl per gram extracted tissue). (may need to heat to 65 °C for 5-10 min. to get into solution)
Add 2 volumes cold 95% EtOH. Invert to mix. Place at -20 °C a few minutes if you can't see DNA precipitate (ppt) form.
Spin 30 seconds at 11 K. Pour off supernatant.
Wash pellet in 0.5 mls 70% EtOH (spin at 11 K, 30 sec.)
Pour off super. Briefly vacuum dry pellet.
Resuspend pellet in 0.1X TE (ca. 200 µl per gm tissue extracted).
Add 1/10 vol RNase stock.
Digest RNA at 37 °C for 1 hour.
Extract with equal volume $CHCl_3$. Spin 11k, 30 sec.
Remove aqueous layer into new tube and add:
0.4 volumes 5M NH_4OAc and 2.5 volumes 95% EtOH.
Invert to mix.
Place at -20 °C for at least 2 hours.
Spin at 12.5 K for 10 min. Pour off supernatant.
Wash pellet with 0.5 mls 70 % EtOH. Spin 12.5 K, 1 min.
Pour off supernatant. Save pellet.
Briefly vacuum dry pellet.
Resuspend pellet in dH_2O.
Estimate DNA concentration by taking OD 260/280 readings of 500 µl of a 1:100 dilutions. (1 OD 260 = 50 µg/ml DNA)

Notes: As vortexing can shear large pieces of DNA, vortexing should be kept to a minimum.
Yield should be about 50 -100 µg DNA per gm of tissue.

VI.5.

Restriction enzyme digestion and electrophoresis of plant DNA

Reagents:

5 M NaOH
5M NaCl
2M Tris-Cl (pH 8.0)
1 M HCl
20 X SSC (see V.5)

6X DNA loading buffer
0.25 % bromophenol blue
30% glycerol

2X TPE buffer (per liter):
8.5 gm Tris
8.0 gm NaH_2PO_4 (monobasic)
0.6 gm EDTA
Add dH_2O to 800 mls. Adjust pH to 7.7
Bring volume to 1L.

Plastic wrap
3mm filter paper
Nitrocellulose paper

Procedures:

Restriction enzyme digestion and electrophoresis of DNA

Digest 10 µg DNA (in 30µl vol. with 5 fold excess of restriction enzyme) 4 hours to overnight.
Add 7 µl 6X DNA loading buffer and load onto 1% Agarose gel (in 1X TPE) and electrophorese in 1X TPE buffer.
Run gel at ca. 75 mAmps.
Stain gel with EtBr and photograph with ruler on UV transilluminator.

Transfer of DNA to nitrocellulose

Before transfer to nitrocellulose, pre-treat gel as follows:
Place gel in dish. Completely cover with the following solutions and shake (50 rpm) at room temp. for the following times:
20 min in 0.25 M HCl
1 hour in 0.5 M NaOH, 1.5 M NaCl
1 hour in 1 M tris (pH 8.0), 1.5 M NaCl

Cut 10 pieces of Whatmann 3mm filter paper and a piece of nitrocellulose (NC) all slightly larger than gel.
Soak NC in dH_2O for 5 min., then in 20X SSC for at least 5 min.
Assemble gel for transfer as follows:
On a piece of plastic wrap place 7 pieces of whatman 3MM paper.
Saturate filter paper with 20 X SSC.
Place DNA gel on top of whatman paper.
Fold plastic wrap up to rest on top of gel, on all sides. This will "seal" the edges of the gel, on all sides.
Place pre-wetted nitrocellulose membrane on top of gel.
Remove all air bubbles by using a 5 or 10 ml pipette like a "rolling pin".
Saturate remaining 3 pieces of Whatman 3mm paper in 20X SSC. Place wet filter paper on top of NC.
Place a stack of paper towels (about 5 inches high) on top of filter paper.
Place a weight (about 500 gms) on top of towels.
Let transfer overnight.

Binding DNA to nitrocellulose (NC).

Next day gently remove NC filter and place between 2 pieces of 3mm paper to air dry.
After the filter has dried, bake at 70 °C in vacuum oven for 30 min.

VI. 6.

Southern blotting: pre-hybridization, hybridization and washing conditions

Reagents:

Pre-hyb buffer:

87.66 gms NaCl
44.12 gms Na Citrate
2.0 gms Ficoll
2.0 gms PVP
volume to 500 mls
Autoclave then add 2 gms BSA.
(Alternative method: add all ingredients and filter sterilize)

1M $NaPO_4$ buffer (pH 6.5)
10 % SDS
Denatured salmon sperm DNA (10 mg/ml)
Formamide

Procedures:

Pre-hybridization:

Place baked nitrocellulose (NC) filter in a seal-a-meal bag with Pre-hyb solution (ca. 20 mls per 150 cm^2 nitrocellulose)
Incubate at 55 °C for at least 2 hours.

Pre-hybridization Solution: (20 mls)

5 mls Pre-hyb buffer
0.4 mls 1M $NaPO_4$ (pH 6.5)
0.5 mls SS DNA (10 mg/ml stock) (boil 5 min before adding)
2.1 mls dH_2O
2 mls 10% SDS
10 mls Formamide

Hybridization of filter:

Either add RNA (ribo-) probe directly to pre-hyb solution or replace with hyb solution containing RNA probe.
Hybridize at 50-55 C for at least 4 hours, with shaking (50 rpm).

Wash conditions:

Cut corner of bag. Drain out radioactive hybridization solution into suitable container.
Gently remove filter from bag. Place filter in dish or other suitable container.
Add enough 2X SSC to cover filter with at least 2 mm of liquid.
Wash in 2X SSC for 15 minutes at room temp on shaker (50 rpm).
Pour off 2X SSC.
Repeat wash procedure (described above) with 0.1X SSC for 40 minutes at 55 °C with shaking (50 rpm).
Pour off 0.1X SSC wash. Check filter with counter. If too "hot" repeat 0.1X SSC wash.
Air dry filter on Whatman 3mm filter paper. Wrap with saran wrap and press to film.

VI.7.

Preparation of single stranded DNA from (M13 ori) plasmids

Reagents:

20 % PEG (MW 8000), 2.5 M NaCl (Autoclave).
L-broth with antibiotics
M13 K07 helper phage stock.
Sterile 1.5 ml Eppendorf tubes
Sterile corex tubes (15 ml)
Phenol
$CHCl_3$
3M NaOAc (pH 5.2) (Autoclaved)
EtOH (95% and 70%)

Procedures:

Day 1:

Inoculate 8 ml of L-Amp (containing 8 µl 1% thiamine) with 40 µl of overnight *E. coli* culture. Use large (ca 40 ml) culture tube.
Incubate at 37 °C, 250 rpm until log growth is reached (ca. 1.5 hours)
Add 50 µl helper phage stock (M13 K07). Incubate at 37°C, 100 rpm 1 hour.
Remove 1 ml of culture. Add to 5-10 mls L-Amp (in large culture tube) with 0.001% thiamine and 70 µg/ml Kanamycin.
Incubate at 37 °C, 250-300 rpm for 14-18 hours.

Day 2:

Transfer culture to 15 ml corex tube, spin 10 min at 8000 rpm.
Transfer supernatant to new 15 ml corex tube.
Add 2.5 mls 20% PEG, 2.5 M NaCl per 10 mls liquid culture volume.
Invert to mix.
Ice 15 minutes.
Spin 10 K for 15 minutes. Pour off supernatant.
Resuspend pellet in 300 µl TE. Transfer solution to eppendorf tube.
Add 300 µl phenol. Vortex. Spin 3 min at 10K.
Transfer Aq layer to new tube. Extract with $CHCl_3$.
Repeat if necessary.
Ether extract (1-2X).
Place aqueous layer in clean tube and add:
1/10th vol 3M NaOAc (pH 5.2) and 2 vols (95%) EtOH.
Invert to mix.
Precipitate at -20 °C ca. 2 hours.
Spin 5 min at 12.5 K in microfuge.

Pour off supernatant.
Wash pellet by adding 0.5 ml 70 % EtOH. Spin at 12 K, 1 min.
Pour off supernatant.
Air dry pellet then briefly vacuum dry pellet.
Resuspend pellet in 20-40 µl TE.
Quantitate DNA concentration by recording OD 260/280 of 500µl of a 1:100 dilution. (1 OD 260 = 40 µg/ml ss DNA)

APPENDIX VII

PROTOPLAST METHODS

VII.1.

Generation of protoplasts from *Nicotiana tabacum*

Reagents:

0.6 M mannitol (autoclaved)
0.6 M sucrose (autoclaved)
10 % MES (pH 5.7 filter sterilized)
Carborundum, cotton swabs, scalpel and forceps.
Sterile dH_20
70% EtOH
10% Chlorox with tween (2 drops per 100 mls).
Cellulase (Onozuka R-10, Yakult Honsha Co. Ltd, Tokyo)
Macerase (Calbiochem)

Procedures:

Surface sterilization of leaf tissue:

Remove mid-vein (use a razor blade) from leaf tissue.
Cut into ca. 1 inch squares with razor blade.
Weigh out 1 gm samples. Place 1 gm in 250 beaker.
Surface sterilize tissue in 200 mls 70% EtOH (90 sec.). Pour off EtOH.
Add 200 mls 10% Chlorox solution. Let stand 5 min.
Pour off Chlorox solution.
Add 100-200 mls sterile dH_2O. Swirl to mix for ca 20 sec.
Pour off dH_2O.
Repeat this dH_2O wash procedure 3-4 X.
Place 1 gm tissue in petri plate.

Enzyme digestion of leaf tissue.

Lightly abrade undersurface of leaf with carborundum on a cotton swab.
Place abraded side of leaf down. Cut into 1 cm. squares using a scalpel and forceps.
Pipette 12 mls enzyme solution into petri dish. Seal with parafilm.
Enzyme solution
0.6 M mannitol, 0.1% MES (Mannitol/MES)
0.15 gms cellulase and 0.02 gms Macerase/ 10 mls.

Let digest overnight, in dark.

Collecting protoplasts:

Next day swirl gently to release protoplasts.
Let digest another 30 minutes to 2 hours in dark.
Filter through sieve with 300 μM diameter openings.
Gently pipette (use a 10 ml pipette) into 15 ml polypropylene tubes.
Underlay 0.6M sucrose cushion (ca. 2 cm thick) in each tube.
    Use a sterile pasteur pipette to underlay sucrose.
Spin 400 rpm (1200 X G, room temp.) 4 min in swinging bucket rotor.
Remove all of the upper phase and the protoplasts at the interface
    with a 10 ml pipette.
Transfer protoplasts to new 15 ml tube.
Spin as above. Protoplasts will pellet in this spin.

Washing protoplasts:

Remove as much of liquid as possible without disturbing protoplasts.
Gently swirl to resuspend protoplasts in remaining liquid.
Gently pipette in 8 mls of Mannitol/MES solution.
Spin as described above. Protoplasts will pellet.
Pipette off liquid with 10 ml pipette.
Swirl to resuspend protoplasts and repeat this wash procedure with
    Mannitol/MES 2 more times.
Count protoplasts in hematocytometer before final spin.
    Add 10-15 μl suspended protoplasts to hematocytometer and
    observe under low power on compound microscope.

VII.2

PEG-mediated transfection of protoplasts

Reagents:

50% PEG 1500 MW (Boehr. Mannheim)
1M $MgCl_2$
1M $Ca(NO_3)_2$ (pH 7-9)
RNA to be transfected
Disposable glass tubes (Kimax 12 x 75 mm), autoclaved.
Mannitol/MES
    0.6 M Mannitol, 0.1 % MES, pH 5.7
0.6 M sucrose
Protoplast incubation media (see II.3)

Procedures:

Transfection:

Pipette 100K protoplasts (in Mannitol/MES) into sterile glass tubes.
Spin down 400 rpm (1200 x G) in swinging bucket rotor,
    4 min at room temp.
Remove as much of supernatant as possible with pipette.
Gently resuspend protos by swirling.
Add RNA directly to protoplasts. Swirl gently.
Immediately add 100 µl PEG-MgCS

PEG-MgCs
    1 ml 50% PEG 1500 MW (Boehr Mannheim)
    15 µl 1M $MgCl_2$
    100 µl 1M $Ca(NO_3)_2$ (pH 7-9)

Swirl gently. Let stand 20 seconds.
Add 1 ml mannitol/MES in 0.33 ml aliquots.
Swirl gently (to dilute PEG) in-between aliquots.
Place tubes in ice 15 minutes.
Spin down (1200 x G, 4 minutes). Remove supernatant with pipette
    and discard.
Resuspend pelleted protoplasts in ca. 0.5 mls incubation media
Transfer to incubation media in small petri dish. (ca. 2.5 mls
    incubation media per 100-200 K protos).
Seal petri plates with parafilm.
Place under lights, cover plates with cheesecloth.

To harvest protos:

Collect protos by gently swirling petri dish (to gently resuspend to dislodge protoplasts).
Pipette protoplasts into 15 ml polypropylene test tube.
Underlay a cushion (ca. 2 cm in height) of 0.6 M Sucrose.
Spin at 400 RPM (1200 x G, swinging bucket rotor, room temp) for 5 minutes.
(Viable protoplasts will collect at and above sucrose/incubation media interface).
With a pipette (10 ml) remove protoplasts in upper phase and at interface. Transfer protoplasts to new tube.
Spin at 400 rpm, 5 min as previously described.
Protoplasts should pellet. Remove all liquid with pipette.
Gently swirl to resuspend. Add ca 5ml Mannitol/MES.
Spin at 400 rpm, 5 min as previously described.
Protoplasts will pellet. Remove all liquid with pipette.
Resuspend protos by gently swirling. Add 0.5 to 1 ml Mannitol/MES.
Count protoplast suspension.
Add 10-15 µl of suspended protos to hematocytometer and observe under low power on compound microscope.

VII.3.

Protoplast printing

Reagents:

Nitrocellulose
primary antibodies (monoclonal or polyclonal)
secondary alkaline phosphatase conjugated antibodies
TBS (see IV.2)
TTBS (see IV.2)

Procedures:

Binding protoplasts to Nitrocellulose:

Pipette ca. 200 protoplasts onto nitrocellulose.
Let air dry.

Detection of bound viral antigen:

Incubate nitrocellulose (NC) in a seal-a-meal bag containing 3% gelatin in TBS* (ca 15 mls per 70 cm^2 NC) for 2 hours at room temp.
Pour out 3% gelatin solution. Replace with an equal volume of primary antibody solution (diluted in 1% gelatin in TTBS*). (example: 1:1000 mouse anti-TEV monoclonals)
Bind primary antibody for at least 2 hours, at room temp.

Washing NC filter:

Remove NC from bag. Place in dish, add enough TTBS to cover with a layer of TTBS at least 2 mm thick.
Place on shaker (50 rpm) at room temp, 7 min.
Pour out TTBS and replace with fresh TTBS.
Repeat TTBS wash procedure 2X.

Binding secondary antibody:

Place NC filter in new seal-a-meal bag with secondary antibody diluted (ex 1:1000) in 1% gelatin in TTBS*. (Use about 15 mls solution/70 cm^2 NC).
Bind secondary antibody at room temp for 1-2 hours.
Remove NC from bag. Place in dish and wash in TTBS as described above (7 min per wash, 50 rpm, etc.). Wash 3X total.
Wash once in TBS on shaker (50 rpm) for 7 min.

Color development:

Place NC in new seal-a-meal bag. Add substrate solution (15 mls per 70 cm^2 NC).

Substrate solution:

100 µl (each) of NBT and BCIP stock solutions per 10 mls carbonate buffer.

Observe blot under binocular microscope (high power) as color develops.

Negative controls should remain as green spots on blot.

Positive controls develop into purple spots.

Stop reaction by removing NC from bag and placing in a dish of dH_2O.

Remove washed NC from dH_2O, place on Whatman 3 mm filter paper to dry. Seal dried NC in seal-a-meal bag to store.

*Note: To get gelatin into solution in TBS and TTBS, it will be necessary to briefly heat the solution in microwave.

APPPENDIX VIII

VIRUS-RELATED PROTOCOLS

VIII.1.

TEV purification

Reagents:

20 mM HEPES (or 200 mM, or 1M stock). Autoclaved.
N-butanol
Sodium sulfite
TEV-infected plant tissue
PEG (MW 8000)
NaCl
Triton X-100
20 mM Tris (pH 8.0)
Sterile corex tubes (15 ml)

Procedures:

Extraction of infected tissue:

Collect Leaf tissue: (usually at least 1000 gms) _____ gms.
Grind tissue in an ice-cold Waring Blendor with:

2X vol:wt 20 mM HEPES (pH 7.5)	(1)_____ ml
18% (final conc.) n-butanol [= (1) x 0.18]	(2) _____ ml
0.1% (wt/vol) NaSulfite [= (1) x 0.001]	(3) _____ gms

Grind on high for 1.5 minutes.
Express through cheesecloth.
Centrifuge in GSA bottles at 4500 RPM for 5 min.
Save supernatant and filter through glass wool. Combine all supernatants.

Precipitation of virus:

Measure volume
volume = _____mls (4)
Transfer to large beaker, add triton, PEG and NaCl.

1.0% (wt/vol)Triton X-100	_____ mls = (4) x 0.01)
4% (wt/vol) PEG 8000	_____ gms =(4) x 0.04)
0.1M NaC	_____ gms =[5.68 x (4), in L]

Stir on ice for 2 hours.
Transfer to GSA bottles, spin 10 min at 8000 RPM.

SAVE PELLETS.
Resuspend pellets well, in 1/4 original volume of buffer used in 20 mM HEPES (pH 7.5) with the aid of a tissuegrinder.
Centrifuge in GSA rotor for 10 minutes at 8000 RPM.SAVE SUPERNATANTS! Combine and measure volume.
volume = _____mls (5)

Transfer to chilled beaker and stir at 4°C for 2 hours after adding:

8% (wt/vol) PEG _____ gms = (5) x 0.08
0.1M NaCl _____gms = 5.68 x (5) in L

Cenrifuge in GSA rotor for 10 minutes at 8000 RPM.
SAVE PELLETS!
Resuspend pellets in 9.0 mls of 20 mM HEPES (pH 7.5) with the aid of a tissue grinder.

Purification on CsCl gradient:

Make up a CsCl solution of the following:

11.6 gms CsCl
27.0 mls 20 mM HEPES (pH 7.5)

To SW 50.1 tubes add:

3.5 mls CsCl solution.
Overlay with 1.5 mls resuspended virus.
Spin at 36,000 RPM for 8-12 hours at 4 °C.

Isolation of virus from Cs gradient:

Virus band should be about 10 mm from the bottom. (Four or 5 mm above this you should see a white floculent band of "junk". Avoid this as much as possible.)
Collect the bands with an 18 gauge needle and 1 ml syringe.
Combine volumes pulled in a 15 ml Corex tube.
Bring the volume to 12 mls with 20 mM HEPES Buffer (pH 7.5).
Centrifuge in an SS34 rotor for 10 minutes at 10,000 RPM.

SAVE SUPERNATANT!
Pour into 50 ml beaker and add PEG to 8% (wt/vol)

PEG _____gms (12 mls x 0.08)

Stir on ice for 1 hour.
Centrifuge for 10 minutes in SS34 rotor at 10,000 RPM, 4°C.
SAVE PELLETS!
Resuspend pellet in 1-2 mls of 20 mM Tris (pH 8.0)

Quantitation of virus recovered:

Record volume of virus solution:

volume = _____mls

Dilute 1:100 into 500 µl dH_2O
Record OD 260/280 of this dilution.

OD_{260} =_____.
OD_{280} = _____.
260/280 = _____ (pure virus 260/280 = 1.18).

To estimate virus concentration:

$$\frac{OD_{260} \times \text{dilution factor}}{2.4} = \text{mg/ml}$$

VII.2.

Purification of TEV RNA

Reagents:

Proteinase K stock solution:

50 mM	Tris-Cl (pH 8.0)
1 mM	$CaCl_2$
1%	SDS
200 µg/ml	Proteinase K

Phenol:$CHCl_3$ (1:1)
5M NH_4OAc
EtOH (95% and 70%)

Procedures:

Proteinase digestion of virus sample:

Incubate proteinase K solution at 50 °C for 10 minutes.
To purified virus sample, add equal volume proteinase K solution.
Vortex briefly to mix.
Incubate at 45-50 °C for 10 minutes.

Precipitation of viral RNA:

Phenol:$CHCl_3$ extract 2X.
Remove aqueous phase and precipitate RNA by adding:
0.4 volumes 5M NH_4OAC
2.5 volumes 95 % EtOH
Invert to mix

Place at -20 °C for 2 hours.
Spin down RNA at 12.5K for 10 minutes in microfuge.
Dump out supernatant. Save pellet
Wash pellet by adding 0.5 ml 70 % EtOH. Spin 12 K, 1 min.
Dump out supernatant.
Briefly vacuum dry pellet.

Quantitation of RNA recovered:

Resuspend RNA pellet in dH_2O.
Estimate concentration by taking OD 260/280 of 500 µl or a 1:100 dilution of RNA solution.

Note: Expect about 25 µg RNA per mg of virus.

VII.3.

Helper component extraction (from PVY infected tissue)

Reagents:

Extraction Buffer (per liter):

Final []	per L
0.1M NH_4 Acetate, pH 8.8,	7.7 gms
0.02M Na_2 EDTA	100 mls 0.2M Na_2EDTA stock
0.02M NaDEICA	4.5 gms

To Ammonium acetate and EDTA solution add dH_2O to bring volume to 1L.
Adjust pH with acetic acid.
Chill at 4°C overnight.
Add Na diethyldithiocarbamate (NaDIECA)just before use.

TSM (per liter):

Tris	12.1 gms
$MgSO_4 \cdot 7H_2O$	4.93 gms

Dissolve in 800 mls dH_2O and adjust pH to 7.2 with H_2SO_4.
Bring final volume to 1L. Autoclave.

40% PEG (wt/vol) solution:

200 gms PEG 6000
500 mls dH_2O
Dissolve PEG in 500 mls dH2O.

Add 40 gms (8%) AG 501-x (Amberlite) mixed bed ion exchange resin (Biorad). Stir overnight at RT. Filter out beads through Whatman 541 filter in a Buchner funnel and vacuum flask. Store at 4°C. (note: deionization is important for consistent active HC preps).

PVY infected leaf tissue (10 gms ca. 2-3 weeks post inoculation).
Miracloth

Procedures:

Collect PVY infected tissue:

Harvest 150g of leaf tissue from plants inoculated with PVY 14-21 days before use.
Place in plastic bag. Chill leaves (4 °C, overnight).
Using a razor blade, Remove the mid-veins and cut into 2 inch squares.

Vacuum infiltrate tissue with Extraction Buffer.

Submerge 100 gms tissue in 1L Buffer in a plastic bag.
Place in dessicator attached to vacuum pump.
Pull a hard vacuum. When the solution starts bubbling close off the dessicator and detach the pump. Release the vacuum to dessicator quickly and watch the water-soaking (darkening) of the tissue.
Repeat this step until all the tissue has turned dark green.
Drain the extraction buffer into a graduated cylinder.

Extraction of HC from tissue:

Place 75 ml of extraction buffer into chilled Blender jar.
Grind the tissue on low speed until all the tissue has been added and blendor runs smoothly.
Homogenize at high speed for 1 min.
Pour homogenized tissue through 4 layers of cheescloth.
Rinse blendor with 25 mls of extraction buffer. Pour rinse through cheesecloth too. Transfer filtrate into GSA bottles.
Centrifuge for 15 min at 8000 g in a GSA rotor.
Pour supernatant through Miracloth (pre-wet with buffer).
Centrifuge filtered supernatant 1.5 hr at 21K in 21 Rotor (or 1 hr at 45 K in 50.2 Ti rotor).
Pour supernatant through wet Miracloth (save 0.5 ml sample for HC activity assay. Store this 0.5 ml sample at -70 °C).

Precipitation of HC:

Save supernatant from above and measure volume.
Add 0.24 ml of 40% PEG Soln. per ml of supernatant (final concentration = 8%).
Stir for 1hr on ice.
Centrifuge 15 min at 8000g. Pour off supernatant. Save Pellets.
Resuspend pellet with glass tissue grinder in TSM (use 1/10 High Speed Supernatant volume of TSM).

Spin (clarify) for 15 min at 8,000g.
Transfer supernatant to clean beaker. (Remove 100 µl sample for assay, store 100 µl sample at -70 °C.)
To remainder of supernatant add 0.24 volumes 40 % PEG.
Stir on ice 1 hour.
Pellet HC with 10 min, 10K spin (SS 34 rotor). Pour off supernatant.
Resuspend pellet in 1-2 mls TSM.
Spin 1 min at 5 K in microfuge to clarify.
Remove supernatant to clean tube and add (dry, powdered) sucrose to a final [] of 20%.
Store frozen (-70 °C) in aliquots (25 - 50 µl).

Test all samples in aphid transmission assay for HC activity (see VII.4). If final sample does not give activity, use the other samples collected along the way to help you identify at which step activity was lost.

VII.4.

Potyvirus purification:
(for aphid transmissable virus)

Reagents:

Homogenization buffer:
0.5 M KPO_4, pH 7.5
1.0 M Urea
0.5% thioglycolic acid (TGA)
0.01 M NaDIECA

Prepare 1L batches of phosphate buffer. Add other ingredients just before use. Mix under hood.
(Note: NaDIECA = Diethyldithiocarbamic acid, sodium salt).

Resuspension buffer:
0.1 M KPO_4, pH 7.0
1.0 M Urea
1 % Triton X-100

20 mM Tris-Cl pH 7.5

53% $CsSO_4$ (wt/wt)

5.3 gms $CsSO_4$
4.7 mls 0.02 M Tris-Cl (pH 7.5)

Potyvirus infected tissue (ca. 2-3 weeks post inoculation).

Procedures:

Collect virus infected tissue the night before. Store at 4 °C overnight in plastic bag.

Extraction of virus from infected tissue:

Homogenize tissue in blendor in 1.5 ml homogenization buffer per gm (fresh wt) of tissue.
Initially add about 1/3 of buffer and then add tissue gradually. Add rest of buffer and homogenize on high ca. 1 min.
Add cold chloroform (1ml chloroform per gm tissue).
Homogenize in blendor 1 minute at low speed.
Transfer slurry to GSA bottles. Spin 15 min at 7K.
Pour aqueous layer through glass wool into chilled graduated cylinder.

Precipitation of virus:

Record Vol: Vol = _____mls
Add NaCl to 0.25 M and PEG (8000) to 4%.

0.0146 X vol = _____ gms NaCl
0.04 X vol = _____ gms PEG

Stir gently at 4 °C for 1 hour.
Transfer to GSA bottles. Spin for 15 min at 7K.
SAVE PELLETS!!
Resuspend pellets in Resuspension buffer.
(Use about 120 mls for 200 gms tissue).
Use glass rod to break up pellets.
Stir gently at 4 °C for 1.5 - 2 hours (make sure all green material is resuspended-- the white junk may never resuspend.)
Transfer to GSA bottles.
Spin 15 min at 7K.
SAVE SUPERNATANT.
Transfer 20 mls of supernatant into 60 Ti tube. Underlay with 5ml sucrose pad (30 % sucrose in 20 mM Tris-CL pH 7.5).
Centrifuge 60 min at 45 K.
SAVE PELLETS.
Resuspend in 0.02 M Tris-Cl pH 7.5 with tissue homogenizer.
(8 mls buffer for 100-200g virus preps).
Transfer to corex tubes. Spin 10 K for 10 min.
Transfer supernatant to new tube.

Separation of virus on Cs gradient:

Record Vol. Add 0.23 gms $CsSO_4$ per ml.
Mix gently to dissolve Cs.
Layer onto 0.8 ml 53% wt/wt $CsSO_4$ pad in 4 ml cellulose nitrate tubes.
(Use P1000 to load onto pad).
Centrifuge 16 hr at 32 K in SW 50.1 rotor.

Isolation of virus:

Pull virus band with large needle and syringe (18 Ga).
Resuspend pulled virus (gently) in ca. 8 mls 20 mM Tris-Cl pH 7.5.
Transfer to corex tube. Spin 10 min at 10K.
SAVE SUPERNATANT.
Transfer super to cellulose nitrate tubes.
Spin in SW 50.1 for 60 min at 45 K, 4 °C.
Resuspend pellets in 0.5 ml 20 mM Tris-Cl (pH 7.5) at 4 °C for 2-4 days with gentle mixing.
Store at 4 °C.

VIII.5

In vitro potyvirus aphid transmission system

Reagents:

Purified PVY helper component (HC)
Purified virus
TSM (with 20% sucrose)

Procedures:

Mix purified HC, virus and TSM (20% sucrose):

Try various ratios of HC and virus to optimize transmission system.
(Ex: Try 1-10% HC, 20-80% virus).

Collect aphids:

Grow aphids on mustard-spinach plants (cv. Tendergreen) in growth chamber with 14 hour photoperiod and 22°/20° (C) day/night temps.
Tap aphids off of leaves into glass dish. Pick up aphids with small paint brush and place into small bottles. Place caps on bottles.
Fast aphids for ca. 1-2 hours.

Aphid feeding:

Stretch parafilm membrane over aphid feeding chambers. (Feeding chambers are 2 cm lengths of 2 cm diameter glass tubing with black electrictrical tape on outside of tubing.)
Place 10-20 aphids on a glass microscope slide.
Place open end of aphid feeding chamber over aphids.
Pipette ca. 20 µl of virus-HC mix on top of parafilm membrane.
Place glass coverslip on top of virus-HC solution.
Let aphids feed on virus-HC solution for 1-2 minutes.
Lift up aphid feeding chamber, remove feeding aphids with a small paint brush.
Place ca. 10 aphids on each test plant (i.e. tobacco seedling).
Let aphids feed overnight then spray with insecticide.
Let virus symptoms develop for at least one week.