Plant Virology Protocols

From Viral Sequence to Protein Function

SECOND EDITION

Edited by

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Plant Virology Protocols

From Viral Sequence to Protein Function
Second Edition

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Preface

Following the considerable success of the Plant Virology Protocols in the Methods in Molecular Biology volume, Humana Press invited us to produce a second edition of this volume.

The first book *Plant Virology Protocols: From Virus Isolation to Transgenic Resistance* had a trend running through it, which people liked, which was – methods to isolate a virus, clone it, express it, and transform it into plants, and evaluate those plants for transgenic resistance.

For the second edition, we have decided on a different trend running through the book that is – *From Viral Sequence to Protein Function*, which will cover the many new techniques that we now can apply to analyze and understand plant viruses.

This book has been divided into five major parts, containing 44 chapters in total.

Part 1 provides a general introduction to some typical plant viral proteins, and their role in infection and interactions with other viral proteins, with the host, vectors, etc.

Part 2 provides a range of techniques for investigating viral nucleic acid sequence as well functional analysis, with Part 3 covering protein analysis and investigation of protein function.

Part 4 has a wide-ranging remit but centered on techniques for microscopy/GFP visualization and analysis/protein tagging/generation of infectious clones and other such tools.

Part 5 covers the emerging area of genomics, interactions with host factors, and plant-based studies, a theme that will probably expand over the coming years to require an entire book dedicated to this theme alone, perhaps *Plant Virology Protocols Vol 3*!

*Plant Virology Protocols* is the product of the hard work and major efforts of a large number of individuals who have been supportive and patient during the editing process. The editors would like to thank them all; we hope they and others will find the book useful and informative.

Gary Foster would like to thank, or should it be apologize to, his family (Diana, James, and Kirsty) for agreeing to take on another book, yes I know I promised I would not take another book, but this one was too nice an idea.

Yiguo Hong would like to thank Gary Foster for inviting him to become involved in this project. Thanks also go to Po Tien, Bryan Harrison, John Stanley, and
Michael Wilson who have consistently inspired him to the tiny but extremely exciting world of plant viruses. Yiguo Hong would also like to thank his family (Mei, Elizabeth, and Lucy) for their support.

Elisabeth Johansen would like to thank Gary Foster for the invitation to participate in the challenging process leading to the publication of this book. Thank you for your guidance and encouragement.

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Chapter 1
Plant–Virus Interactions

Peter Palukaitis, John P. Carr, and James E. Schoelz

Abstract A variety of techniques have been used to examine plant viral genomes, the functions of virus-encoded proteins, plant responses induced by virus infection and plant–virus interactions. This overview considers these technologies and how they have been used to identify novel viral and plant proteins or genes involved in disease and resistance responses, as well as defense signaling. These approaches include analysis of spatial and temporal responses by plants to infection, and techniques that allow the expression of viral genes transiently or transgenically in planta, the expression of plant and foreign genes from virus vectors, the silencing of plants genes, imaging of live, infected cells, and the detection of interactions between viral proteins and plant gene products, both in planta and in various in vitro or in vivo systems. These methods and some of the discoveries made using these approaches are discussed.

Keywords Agroinfection; Agroinfiltration; Green fluorescent protein; Plant gene isolation; Microarrays; PR proteins; Resistance responses; Salicylic acid; Transgenic plants; Virus-induced gene silencing

1 Introduction

Research in plant virology has consisted predominantly of studying diseases induced by plant viruses and characterizing the viruses involved in various diseases of plants (reviewed in ref. 1). While physiological responses in plants to infection by viruses have been measured for many years using biochemical methods, it is only in the last 20 years that plant–virus interactions have been analyzed at the molecular, cellular, and genetic levels (1, 2). A variety of techniques that allowed the characterization of the interactions between plants and viruses has become available. These techniques are described in the following chapters, as are various functions and processes involved in virus–virus, virus–host, and virus–vector interactions.

Prior to the mid 1980s, the technology available allowed only limited molecular analyses of plant–virus interactions. Many of these analyses concerned determining...
the genome organization of viruses and the nature of the gene products of viral genomes. In vitro translation was used to assess the number of gene products and the modes of gene expression of plant viruses with plus-sense RNA genomes (reviewed in ref. 3). Because plant viruses do not inhibit the expression of most plant genes, the in vivo detection of viral-encoded gene products was limited largely to the highly expressed capsid protein. Protoplasts prepared from mesophyll cells of leaves were used to study the kinetics of virus replication, as well as whether resistance genes had any effect on virus replication (reviewed in ref. 4); a method still in use today. For viruses with divided genomes, separation of the individual genomic components and reassortment (also referred to as pseudorecombination) were used to map phenotypes, such as symptomatology, and host range to specific genome segments and sometimes to individual genes. However, localization of phenotypic changes to specific nucleotides required the development of cloning techniques for viruses with RNA genomes, in vitro RNA transcription of infectious genomes from cloned cDNAs, sequence determination of the genomes, and mutagenesis methods for modifying specific nucleotides in cloned cDNAs (5–9). These techniques had all been developed by the mid 1980s, although many refinements have been made since then.

2 Approaches to Virus Genome and Gene-Function Analyses

The development of various cDNA cloning techniques has allowed representatives of virtually all viral genera to be cloned and their genome sequences to be determined. This has allowed the number, position, and mode of expression of the various genes to be determined (10). Mutagenesis methods have allowed the roles of various genes and their encoded proteins to be determined, and also whether these genes or sequences support replication, movement, transmission, or act as elicitors of defense functions, and more recently, whether they act as counter defense proteins (e.g., suppressors of RNA silencing) (reviewed in ref. 1). Sequence analysis also has been useful in identifying conserved domains and potential active sites of proteins. Sequencing of strains of the same virus for identifying target sequences combined with mutagenesis has allowed the identification of specific viral sequences involved in eliciting various responses between plants and viruses. These methods have not, however, allowed identification of the host components involved in these interactions. By contrast, various approaches developed and used in the last 20 years have allowed some of these host factors and their functions to be identified.

2.1 Gene Exchange to Localize Determinants

As infectious clones of plant viruses have been developed, gene-exchange experiments have been the preferred method for identification of avirulence (Avr) and symptom determinants. The advantage of genetic exchange between two closely
related strains is that this allows one to test the effect of viral genes in the context of an infection in a whole plant. All that is required is to have infectious clones of two virus strains that differ in one or more biological properties. In the early days, it was important to locate restriction enzyme sites common to the two virus strains to engineer the exchange, but with the advent of PCR, exchanges can now be made at any point in the virus genome. Reciprocal exchanges are best, because they allow for a more thorough examination of a specific trait: one chimera can be used to prove that a specific viral gene is an Avr determinant, whereas the reciprocal chimera shows that no other viral gene product acts as a second Avr determinant in a given host.

The first virus Avr gene product identified through gene exchange was P6 of cauliflower mosaic virus (CaMV) (11, 12), a CaMV protein that interacts with host ribosomes to reprogram them for expression of the polycistronic CaMV 35 S RNA (13, 14). P6 triggers a hypersensitive response (HR) in Datura stramonium and Nicotiana edwardsionii, and a nonnecrotic resistance response in Nicotiana bigelovii. The same studies also showed that P6 is an important symptom determinant, as it is a primary determinant of chlorosis in crucifers (11). The first Avr gene product of an RNA virus to be characterized by gene exchange was the coat protein of tobacco mosaic virus (TMV) (15, 16). As these types of studies have accumulated, it is has been found that virtually any type of viral gene product may trigger a resistance response in plants (17).

2.2 Viral Protein Function Analyses and Localization

The overexpression of viral-encoded proteins in Escherichia coli, often tagged with either six histidines (His6) or glutathione transferase (GST) to aid in their purification, has been very useful in producing proteins that can be used to study various in vitro functions of such proteins. These include binding to other viral-encoded proteins (using GST-pull down assays, coimmunoprecipitation, filter binding assays, or surface-plasmon resonance) or to nucleic acids (18–22). These proteins also have been used to produce antisera that were then used to detect these proteins and localize them in situ by immunogold labeling and electron microscopy (23, 24). E. coli expression of viral-encoded proteins has largely superseded expression of such proteins in insect cells, which also has been used.

2.3 Plant Genetic Approaches

Different plant varieties, cultivars, ecotypes, and accessions have been used for identifying viral processes affected by changes in host genes, especially the effects of resistance genes on infection. A number of dominant resistance genes have been isolated, first using insertional mutagenesis and later map-based cloning approaches. These include the resistance genes N (against TMV) from tobacco (25), Rx [against
potato virus X (PVX)] from potato (26, 27), Tm2 (28) and Tm22 (29) (against tomato mosaic virus), as well as Sw-5 (30) (against tomato spotted wilt virus) from tomato, and RCY1 (31) (against cucumber mosaic virus), as well as RTM1 and 2 (32, 33) (against tobacco etch virus) from Arabidopsis thaliana. A number of recessive resistance genes also have been identified from A. thaliana and various crops species, using candidate gene mapping approaches, demonstrating that the genes encoding translation factors eIF4E, eIF(iso)4E, and eIF4G are involved in resistance to viruses in various groups (reviewed in ref. 34). A. thaliana has proven to be particularly useful for isolating genes affecting virus infection, for several reasons, including the small genome size, the large number of ecotypes and mutant genomes available, and especially because the entire genome has been sequenced and annotated.

The study of resistance and host reactions to virus infection has, like most areas of plant virology, been technology driven. Thus, as new techniques become available, dormant topics have become accessible to further investigation. Attempts to better understand host reactions or resistance to plant viruses have in some instances revealed important aspects of viral biology; for example, in the study of recessive genes conferring resistance to potyviruses (reviewed in ref. 34). Paradoxically, the discovery of novel host responses to viruses has sometimes turned out to be more useful for our understanding of resistance to nonviral pathogens, as exemplified by the discovery and subsequent studies of the pathogenesis-related (PR) proteins.

3 Plant Responses Induced by Virus Infection

The work of Ross and Kuć and their respective colleagues in the 1960s and 1970s on induced resistance prompted several research groups to seek new proteins or other factors associated with resistance induction (reviewed in ref. 35). Induced resistance, most commonly referred to as systemic acquired resistance (SAR), is the enhancement of a plant’s resistance to disease triggered by previous exposure to an avirulent pathogen.

3.1 “Novel” Proteins: The Discovery of IVR and PR Proteins

Loebenstein and coworkers exploited protoplast techniques to identify an extracellular protein produced by plants following a TMV-induced HR. The HR is a resistance response usually characterized by programmed host-cell death in the vicinity of pathogen entry and often followed by the induction of SAR. This protein inhibited accumulation of several viruses in leaf discs and so was called an inhibitor of virus replication (IVR) (36, 37). Recently, it was shown that constitutive expression of an IVR cDNA in transgenic plants provided some protection against virus infection and, surprisingly, against a fungus (38). The results appear to confirm that IVR may play a role in induced resistance to viruses but they also raise some interesting
new questions regarding the mode of action of a factor that can inhibit the life cycles of pathogens as diverse as viruses and fungi.

Probably the most intensively studied inducible gene products associated with the HR and SAR induction are a very diverse set of proteins, the PR proteins. The first PR proteins to be identified were acidic, extracellular proteins belonging to the PR1 and PR2 families (39). PR proteins were discovered independently by van Loon and Gianinazzi, together with their respective colleagues, by comparing the protein compositions of healthy tobacco plants with those of plants infected systemically with TMV or exhibiting the HR in response to the virus (40, 41). Both groups detected “novel”, host-encoded proteins accumulating in the leaves of NN genotype (TMV-resistant) tobacco plants inoculated with TMV and in leaves expressing SAR. PR proteins are defined now as any plant proteins induced as a result of pathogen infection or attempted infection and many different families have been defined (42). Certain PR proteins, notably members of the PR1 family, and their mRNA transcripts have proved to be very useful molecular indicators for the induction of SAR (43, 44). However, their levels can also increase during systemic infection by certain viruses (45). Furthermore, albeit a correlative relationship between PR1 protein or gene induction and resistance to viruses was suggested in early work (46), constitutive expression of PR1 and several other PR proteins in transgenic plants did not result in enhanced resistance to viruses (47, 48), although it did provide protection against fungal infection (49). Thus far, none of the proteins defined as “PRs” are known to have any antiviral effects. However, it might be argued that a plant RNA-dependent RNA polymerase, RdRp1, which is induced by salicylic acid (SA) and during systemic virus infection and promotes sequence-specific turnover of TMV RNA in tobacco (50), could be considered a PR protein under the current rather wide definition.

Serendipitously, for their pioneering work on PR proteins, Gianinazzi and van Loon both used the native polyacrylamide gel electrophoresis (PAGE) system of Ornstein (51) and Davis (52). Using this system, the highly charged, acidic PR proteins are the most conspicuous bands in the electrophoretic patterns produced on 10% acrylamide gels by proteins extracted from hypersensitively responding tobacco leaves. On one-dimensional SDS-PAGE gels, PR protein bands are completely obscured by a background of polypeptide bands representing constitutive plant proteins. Without the fortuitous use of native PAGE to examine changes in protein composition accompanying the HR, much of the field of molecular plant pathology would have taken a rather different course and our knowledge of plant responses to infection, including mechanisms of resistance to fungal and bacterial infection, the defensive signaling roles of SA, and the regulation of defense genes, would have taken longer to acquire.

### 3.2 Defensive Signaling: Changes in SA Accumulation

White (53) showed that treatment of plants with aspirin (acetylsalicylic acid) solutions induced SAR and PR protein accumulation in tobacco. This prompted the suggestion that a benzoic acid or salicylic acid-like signal chemical might play a
role in the establishment of SAR, following a HR (54). This idea was vindicated through the use of sensitive analytical technologies, which showed that SA levels increase dramatically following a virus or fungus-induced HR (55, 56), as well as the application of plant transformation to create transgenic plants that were unable to accumulate SA (due to constitutive expression of an SA-degrading enzyme) and consequently were unable to exhibit HR-type resistance or SAR against viral and other pathogens (57).

The analytical techniques used for the detection of SA in infected plant tissue were developed from earlier studies on the role of this chemical in triggering heat production in thermogenic plants (58), and most assay methods that have followed take similar approaches. These measurements typically involve extraction of phenolic compounds from plant tissue followed by high performance liquid chromatography (HPLC) and fluorescence detection of the SA peak. In some studies, the presence of SA has been further authenticated by mass spectrometry (55, 56, 58). This type of procedure can be adapted for high-throughput analysis of many samples at once (59).

The HPLC methodology is highly accurate and quantitative, but destructive, and provides no information on the distribution of SA in living tissues. To address this problem, a new method has recently been developed in which genetically engineered, nonpathogenic bacteria (Acinetobacter sp. ADP1) harboring an SA-responsive lux reporter gene construct are infiltrated into the leaf apoplast or stems of plants following viral challenge. In NN genotype tobacco responding to TMV infection, the accumulation of SA around the developing HR lesions could be imaged utilizing the SA-induced bioluminescence from the engineered bacteria (60). By calibrating the imaging system using known amounts of SA into plant leaves, it was possible to make quantitative determinations of SA across the tissues (60).

3.3 Temporal and Spatial Plant Responses to Infection

The technology of the period 1970 to ca.1995 limited the examination of “molecular” markers, that is, proteins and RNA transcripts, for host reactions to virus infection to a relatively hit-or-miss approach. This has changed significantly with the advent of “-omics” approaches in which it is potentially possible to monitor changes in levels of hundreds or thousands of transcripts, proteins, or metabolites in virus-infected plant tissue. For example, using A. thaliana DNA microarrays, Whitham and colleagues (45) identified transcripts that increase or decrease in their steady state levels in response to systemic infection by any of five different viruses. In contrast to this group of nonspecifically responding transcripts, levels of certain other host mRNAs are changed only in response to certain specific viruses. For example, the PRI gene and several other SA-regulated genes were upregulated by infection with a potyvirus and a cucumovirus but not by infection with viruses belonging to three other genera (45).
Nevertheless, this powerful technology may not be very useful in interpreting the responses of host cells actually infected with the virus. Even with the most concentrated of virus inocula, only about 0.1% of the epidermal cells in directly inoculated leaves initially become infected (61) and although the proportion of virus-harboring cells in systemically infected tissue will be much higher, it will be a mixed population of virus-infected and noninfected cells. Therefore, the results of the current generation of microarray studies using RNA samples taken from systemically infected plants need to be interpreted with caution. In the future, emerging technologies such as single-cell sampling techniques (62), combined with the use of green fluorescent protein (GFP)-expressing viruses and microarray techniques requiring smaller amounts of RNA, are likely to allow the very precise assessment of changes in host metabolism and host and viral gene expression in different host cells at different stages of virus infection.

Using currently available technology, virus-induced alterations in metabolism and gene expression are best studied in the earliest phases of infection using directly inoculated tissue because this shows the greatest degree of synchrony. This has been exploited to greatest effect by Maule and collaborators using in situ hybridization techniques to detect host and viral RNAs and microanalytical methods to assess biochemical changes in virus-infected pea seeds and marrow (squash) cotyledons, respectively (63–66; also discussed in ref. 1).

4 Approaches to Study Plant–Virus Interactions

4.1 Development of Transgenic Plants

It has now been over 20 years since the first publications describing the development of transgenic plants, a technique that has truly revolutionized how plant biology research is conducted. In 1983, four groups published their evidence that they had successfully developed transgenic plants. The transgene of choice for three of the four groups was a kanamycin resistance gene, although it was expressed in three different types of plants: petunia, cultivated tobacco (*Nicotiana tabacum*) and a close relative of tobacco (*Nicotiana plumbaginifolia*) (67–69). The fourth group chose to express a bean protein in sunflower (70).

Virologists quickly recognized the practical value of transgenic technology, as it could be used for development of new methods for controlling virus diseases. For example, one of the first applications of transgenic technology involved expression of the TMV coat protein in transgenic tobacco, which protected plants from infection by TMV (71). This technology, called pathogen-derived resistance, has been utilized to protect many types of crop plants from a broad variety of viruses (72). Other transgenic techniques also have been developed; in particular, methods based on homology-dependent gene silencing (now known as RNA silencing). Furthermore, as host virus resistance genes have been cloned, it has been possible to move them across species barriers, as was done in moving the *N* gene from tobacco to tomato (73).
Transgenic plants also have been utilized for basic studies on how viruses cause disease and the strategies plants use to protect themselves from pathogen attack. As virus genes were converted into transgenes for expression in plants, it was discovered that some could elicit symptoms. The first example was P6 of CaMV, which elicited virus-like symptoms in tobacco when expressed as a transgene (74). Viral transgenes also have been used for the development of elegant complementation systems. For instance, transgenic tobacco that expressed the movement protein of TMV could complement movement-defective strains of TMV (75). In addition, attempts to express both wild type and mutant forms of viral transgenes also contributed to the discovery of RNA silencing mechanisms in plants (76, 77) and the discovery of virus-encoded silencing suppressors (78–80). It is now known that several viral genes that function as suppressors of RNA silencing also have the capacity to induce symptoms in plants when expressed as a transgene.

4.2 Virus Gene Expression and Silencing Vectors

Viral-derived gene expression vectors have been used with great success to probe the functions of genes involved in plant defenses. Over the past 10 years, it has become apparent that viruses themselves can be the targets of the plant’s gene silencing apparatus. By inserting host nucleotide sequences into a virus vector, researchers can trick the plant into degrading its own mRNAs. Host nucleotide sequences carried in a virus vector will be targeted for degradation, but most importantly, plant mRNAs homologous to the sequence in the virus also will be degraded. This technique is called virus-induced gene silencing (or VIGS). In a classic experiment, Ruiz et al. (81) created a PVX vector that carried portions of the plant gene, phytoene desaturase. When this PVX vector was inoculated to plants, the endogenous phytoene desaturase mRNA was targeted for degradation along with the virus, and this resulted in photobleaching in the leaves. VIGS has been used to silence host resistance genes (82) and also has been used in high-throughput assays to characterize plant genes required for the plant defense response (83).

In addition to characterization of host genes necessary for plant defenses, virus vectors also have been useful for identification and characterization of viral Avr genes. In particular, they can be used when no known resistance-breaking strain has been found. For example, all known tomato bushy stunt virus (TBSV) strains elicit HR in N. tabacum and N. edwardsonii; consequently, gene exchanges are not an option. Scholthof et al. (84) inserted TBSV genes individually into a PVX vector and found that TBSV proteins P19 and P22 were Avr determinants. P19 triggered HR in N. tabacum, whereas P22 triggered HR in N. edwardsonii. Furthermore, this system could be used to dissect the functions of Avr proteins from other functions associated with either P19 or P22; experiments that could not be done in the context of an infectious virus clone. For example, a PVX vector was used to show that the cell-to-cell movement function of P22 could be separated from its capacity to elicit HR in N. edwardsonii (85).
4.3 Imaging of Infected Cells

Nondestructive imaging of virus movement through plant tissues at the micro- and macroscopic levels has been possible since the mid-1990s, owing to the development of genetically modified viruses expressing GFP and other fluorophores (86). Experiments with GFP-expressing viruses and viral protein–GFP fusions, especially when combined with imaging using the confocal scanning laser microscope, have revolutionized our understanding of virus movement in susceptible hosts and the mechanisms of intercellular communication in plants (reviewed in ref. 87). Application of these technologies to the investigation of viral movement and distribution around HR lesions and in SA-treated tissues has raised new questions regarding the involvement of mechanisms such as RNA silencing in induced resistance and how antiviral mechanisms may differ between different cell types in the same plant (88, 89).

4.4 Agroinfection and Agroinfiltration

Agroinfection (also known in the literature as agroinoculation) is a technique in which an infectious clone of a virus is inserted into the T-DNA present on the Ti plasmid of Agrobacterium tumefaciens. A. tumefaciens is subsequently used to deliver the infectious viral DNA into a plant cell, where it is released from the T-DNA and the infection is initiated. Agroinfection was first demonstrated with the caulimoviruses and geminiviruses (90, 91) and is now the primary method for initiating infections with geminivirus and luteoviruses (92, 93). Agroinfection is also increasingly being used for inoculation of infectious clones of RNA viruses (82, 83, 94–97). In this variation, the viral cDNA is expressed from a constitutive promoter such as the CaMV 35 S promoter. Once the viral cDNA is delivered to cells by A. tumefaciens, the host RNA polymerase II will utilize the plant promoter to initiate synthesis of an infectious RNA. As with the DNA viruses, agroinfection of infectious cDNAs based on RNA viruses is much more efficient and cost effective, as it eliminates the need for in vitro transcription.

Agroinfiltration is a variation in which individual viral genes are expressed in a transient fashion (98). Agroinfiltration provides a rapid alternative for screening Avr genes compared to expression in viral vectors or gene swaps between infectious virus clones. In this technique, a putative Avr gene is placed under the control of a promoter such as the 35S promoter and the cassette inserted into the T-DNA of the Ti plasmid of A. tumefaciens. A. tumefaciens containing this Ti plasmid is treated with acetosyringone, which mobilizes the transfer of the T-DNA, and after 24 h the cells are infiltrated into the leaf. Plant tissues infiltrated with an Avr gene will develop the HR at a rate comparable to a virus-inoculated plant. Agroinfiltration has been used successfully to illustrate the function of Avr genes from several viruses. For example, the TMV replicase elicits HR when agroinfiltrated into N-gene tobacco (99, 100) and the coat protein of PVX elicits HR when agroinfiltrated into potatoes containing Rx gene (26).
As with virus vectors, agroinfiltration has two advantages over gene exchange experiments with infectious virus clones. First, it allows for the identification of an \textit{Avr} gene when no resistance breaking strain of the virus is available. Second, it is possible to isolate domains of \textit{Avr} proteins capable of eliciting HR. For example, agroinfiltration was used to show that the helicase domain of the TMV replicase protein was responsible for eliciting the HR in \textit{N}-gene tobacco (99, 100). Agroinfiltration also has been developed as a tool for initiation of gene silencing in plants (98) and in the identification of virus suppressors of gene silencing (101).

4.5 \textit{Interactions between Host and Viral Molecules}

The outcome of many interactions between viruses and their hosts (susceptibility, resistance, or no interaction) is likely to be influenced at some point by the intermolecular interactions of host and viral proteins. Such protein–protein interactions are difficult or impossible to identify \textit{in planta} and so potentially interacting plant and viral proteins have been investigated in vitro or under physiological conditions using the yeast two-hybrid system. In the yeast two-hybrid system, sequences from pairs of candidate interacting proteins are, respectively, engineered to form translational fusions with two separate parts of an artificial transcription factor. If the candidate interacting protein sequences do indeed bind to each other, this will bring together a functional transcription factor that will activate reporter gene activity in the yeast cell (102). The yeast two-hybrid approach is fraught with technical problems but can be extremely informative when its conclusions are backed up by other approaches, for example, in vitro interaction assays using coimmunoprecipitation or analysis of interactions between mutant plants or viruses (see Sects. 2.2 and 2.3).

The best case study in which this combination of approaches has yielded important biological data is in the interaction between the potyviral VpG proteins and the translation factors eukaryotic initiation factor eIF4E and eIF(iso)4E in various hosts (reviewed in ref. 34). The interaction is required for successful infection by potyviruses and underpins most examples of recessive resistance to potyviruses in a wide range of plants including Arabidopsis, pea, tobacco, tomato, pepper, lettuce, and various Brassica species, resistance results in each example from expression of a modified translation factor, with which the potyviral VpG is unable to interact (34, 103–106).

5 \textit{Concluding Remarks: Future Directions}

Over the past 25 years, infectious clones and virus genome sequences have become a prerequisite for the study of plant viruses. For most viruses, the primary functions for each of their genes have been determined (i.e., coat protein, movement protein, replicase) and secondary and tertiary functions (i.e., silencing suppressor, symptom